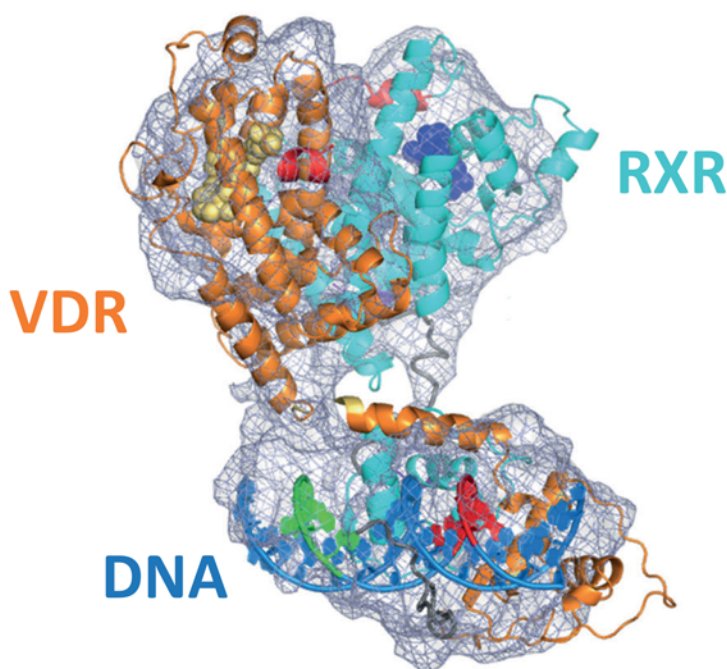


Feldman and Pike's  
**Vitamin D**

*Celebrating 100 Years of Vitamin D Research*



**VOLUME TWO:**

**Disease and Therapeutics**

Edited by  
Martin Hewison, Roger Bouillon  
Edward Giovannucci, David Goltzman  
Mark Meyer, JoEllen Welsh



FELDMAN AND  
PIKE'S VITAMIN D

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# FELDMAN AND PIKE'S VITAMIN D

## Volume Two: Health, Disease and Therapeutics

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### FIFTH EDITION

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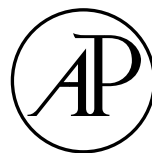
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# Contents

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List of contributors	xxvii
In memoriam	xxxv
Preface to the fifth edition	xli
List of abbreviations	xliii
Relevant lab values in adults and children	li

## VOLUME I

### 1. Book introduction: Major trends in vitamin D research

DAVID FELDMAN AND J. WESLEY PIKE

1. History of the <i>Vitamin D</i> book	1
2. Major trends in the vitamin D field	1
3. Mechanism of action	2
4. Mutations in the VDR and critical metabolic enzymes cause childhood rickets	3
5. Vitamin D assays	4
6. Extra-skeletal actions	4
7. Population studies and clinical trials	4
8. Controversy: what constitutes normal or adequate concentrations of vitamin D?	5
9. Vitamin D and autoimmune disease	5
10. Vitamin D and COVID-19	5
11. Vitamin D and cancer	5
12. Is vitamin D useful for the prevention or treatment of colorectal cancer?	6
13. Closing comments with thanks and appreciation to the many authors, the critical Elsevier staff, and the many readers that made this undertaking possible	7

References

### 2. Evolution of human skin pigmentation and vitamin D

NINA G. JABLONSKI

1. Introduction	9
2. The solar context of human skin color evolution	10
3. The nature of the selective factors leading to the evolution of skin pigmentation in early <i>Homo</i>	11
4. Folate conservation and the evolution of permanent protective pigmentation	12
5. Early hominin dispersal out of the tropics and vitamin D production	12

6. The evolution of skin pigmentation in <i>Homo sapiens</i>	16
7. The effects of cultural buffering, rapid migrations, and modern lifestyles on skin color and vitamin D	19
8. Summary points	20
References	21
Further reading	25

### 3. Photobiology of vitamin D

MICHAEL F. HOLICK AND  
ANDRZEJ T. SLOMINSKI

1. Introduction	27
2. Historical perspective	28
3. Photobiology of vitamin D	29
4. Role of sunlight and dietary vitamin D in bone health, overall health, and well-being	33
5. Ultraviolet devices for producing vitamin D	34
6. Alternative pathways generating noncanonical active forms of vitamin D and their photoderivatives	35
7. Sunlight, vitamin D, and skin cancer	36
8. Conclusions	37
9. Summary points	39
Acknowledgment	39
References	39
Further reading	45

### 4. The activating enzymes of vitamin D metabolism (25- and 1 $\alpha$ -hydroxylases)

GLENNVILLE JONES, DAVID E. PROSSER, AND  
MARTIN KAUFMANN

1. Introduction	47
2. General information regarding vitamin D hydroxylases	49
3. Vitamin D <sub>3</sub> -25-hydroxylases	49
4. 25-Hydroxyvitamin D-1 $\alpha$ -hydroxylase	57
5. Additional topics	60
6. Conclusions	64
7. Summary points	64
References	65
Further reading	69

## 5. CYP24A1: structure, function, and physiological role

RENÉ ST-ARNAUD AND GLENVILLE JONES

1. Introduction	71
2. CYP24A1-catalyzed pathways	71
3. C24-oxidation pathway	72
4. C23-hydroxylation pathway	73
5. CYP24A1, a multifunctional enzyme	73
6. Biological relevance of the C24-oxidation pathway	74
7. Structure—function relationships	74
8. Mutations of CYP24A1 in infantile hypercalcemia type 1	76
9. Preclinical models of infantile hypercalcemia type 1	77
10. CYP24A1 involvement in other systems	77
11. 24,25(OH) <sub>2</sub> D <sub>3</sub> and chondrocyte maturation	77
12. 24,25(OH) <sub>2</sub> D <sub>3</sub> and fracture repair	78
13. Conclusions	79
14. Summary points	80
References	80

## 6. Alternative pathways for vitamin D metabolism

ANDRZEJ T. SLOMINSKI, ROBERT C. TUCKEY, CARL JENKINSON, WEI LI, AND ANTON M. JETTEN

1. Introduction	85
2. Cytochrome P450 <sub>scc</sub> (CYP11A1): an overview	86
3. Introduction to the skin in a photobiology context	86
4. CYP11A1 mediated metabolism of 7DHC with local and systemic implications	88
5. CYP11A1-mediated metabolism of vitamin D	88
6. Enzymatic activation of lumisterol and tachysterol	92
7. Phenotypic activity of CYP11A1-derived hydroxyderivatives and their mechanism of action	92
8. C3-epimerization of vitamin D metabolites	97
9. Phase II conjugation of vitamin D metabolites	99
10. Conclusions	103
11. Summary points	103
Acknowledgment	103
References	103

## 7. Vitamin D—binding protein

ROGER BOUILLON, RENE F. CHUN, AND FRANS SCHUIT

1. Introduction	111
2. Vitamin D-binding protein: gene and protein structure	112
3. Functions of vitamin D—binding protein	118
4. The vitamin D—binding protein—actin complex	126

5. DBP binds fatty acids	128
6. The role of vitamin D—binding protein in inflammatory processes and in the immune system	128
7. Conclusions	129
8. Summary points	130
References	130

## 8. Regulation of renal vitamin D metabolism

MARK B. MEYER, NANCY A. BENKUSKY, SEONG MIN LEE, AND J. WESLEY PIKE

1. Introduction	139
2. Major physiologic regulators of renal vitamin D metabolism	140
3. Genomic structure and deletion of the <i>CYP27B1</i> and <i>CYP24A1</i> genes	141
4. Genomic mechanisms for <i>CYP27B1</i> regulation	142
5. Genomic mechanisms for <i>CYP24A1</i> regulation	148
6. Conclusions	149
7. Summary points	151
Acknowledgments	151
References	151

## 9. Regulation of extra-renal synthesis of 1,25(OH)<sub>2</sub>D

MARTIN HEWISON AND JOHN S. ADAMS

1. Introduction	155
2. 1 $\alpha$ -OHase and the <i>CYP27B1</i> gene	156
3. Macrophage 1 $\alpha$ -OHase	157
4. Macrophage 1 $\alpha$ -OHase and responsiveness to immune cell regulators	161
5. Molecular mechanisms for regulation of extrarenal 1 $\alpha$ -OHase	164
6. 1 $\alpha$ -OHase in cells other than macrophages	167
7. Dysregulated synthesis of 1,25(OH) <sub>2</sub> D and human disease	173
8. Conclusions and future prospects	176
9. Summary points	178
References	178

## 10. Structural basis for ligand activity in vitamin D receptor

ANNA Y. BELORUSOVA AND NATACHA ROCHEL

1. Introduction	189
2. Conclusions and perspectives	207
3. Summary points	207
References	208

## 11. Genome-wide perspectives on vitamin D receptor actions

J. WESLEY PIKE, SEONG MIN LEE, NANCY A. BENKUSKY, AND MARK B. MEYER

1. Introduction	213
-----------------	-----

2. Target genes involved in the regulation of mineral metabolism	213	7. Interplay between FoxO proteins and the VDR	247
3. The production of 1,25(OH) <sub>2</sub> D <sub>3</sub> in diverse nonrenal target cells	214	8. Vitamin D signaling regulates the function of the oncogenic transcription factor c-MYC	249
4. Transcriptional mechanism of action of 1,25(OH) <sub>2</sub> D <sub>3</sub>	214	9. Widespread crosstalk between NF-κB and vitamin D signaling	251
5. New insights into transcriptional activation of vitamin D	216	10. Insights into VDR-dependent transcriptional regulation from genomics studies	252
6. Current advances in transcriptional regulation	218	11. Conclusions	253
7. Analogs of vitamin D	218	12. Summary points	253
8. Pharmacology of vitamin D analogs	219	References	253
9. Recent possibilities for enhancing vitamin D analog SAR potential	220	Further reading	259
10. Highlights of transcriptional mechanisms	220		
11. Returning to vitamin D analogs—a new analytical approach	224	<b>14. Vitamin D and microRNAs</b>	
12. Conclusions	224	HENGGUANG ZHAO, MARIANELA FORCELLATI, DAMIEN BUSCHITTARI, JOHN E. HECKEL, CHRISTOPHER J. MACHADO, SRI RAMULU N. PULLAGURA, AND THOMAS S. LISSE	
13. Summary points	224		
Acknowledgments	225	1. Introduction	261
References	225	2. MicroRNAs: the posttranscriptional age	263
		3. Complexity of miRNA regulation, biogenesis, and posttranscriptional regulatory functions	263
<b>12. Vitamin D, chromatin, and epigenetics</b>		4. Nuclear receptors and miRNAs	266
CARSTEN CARLBERG		5. The relationship between miRNAs and the vitamin D synthesis, metabolism, and signaling systems	267
1. Introduction	229	6. Interplay between vitamin D and miRNAs in biological systems	274
2. The impact of chromatin	230	7. Translational aspects of miRNAs	281
3. The impact of epigenetics	231	8. Strategies for miRNA and target identification for vitamin D research	282
4. The nuclear receptor superfamily member VDR	232	9. Conclusions	284
5. Genome-wide location of VDR-binding sites	232	10. Summary points	284
6. CTCF as organizer of vitamin D-dependent chromatin domains	234	Acknowledgments	284
7. Vitamin D-triggered epigenetic changes	234	References	284
8. A chromatin model of vitamin D signaling emerges	235		
9. Epigenetic effects of vitamin D in vivo	235	<b>15. Pathway based-analysis of transcriptomic data from vitamin D studies</b>	
10. Conclusion	237	CHRIS T.A. EVELO AND SUSAN L.M. COORT	
11. Summary points	237		
References	237	1. Introduction	291
Further reading	239	2. Transcriptomic data	291
		3. Vitamin D <sub>3</sub> -related pathways in online pathway databases	291
<b>13. The diverse genomic mechanisms of action of the vitamin D receptor</b>		4. Workflow pathway-based analysis for transcriptomic data	293
JOHN H. WHITE, FATEMEH SARMADI, AND PATRICIO ARTUSA		5. The six steps in the pathway-based analysis workflow of transcriptomic data	293
1. Overview	241	6. Example case—immunometabolic effect of vitamin D <sub>3</sub> in dendritic cells and monocytes	296
2. The VDR and the hairless repressor: regulation of transcription in the absence of 1,25(OH) <sub>2</sub> D	242	7. Conclusion	297
3. Crosstalk between the VDR and TGF-β signaling	243	8. Summary points	298
4. The VDR and the AP-1 family of transcription factors	244	References	298
5. JAK/STAT signaling and the VDR in immunity	244		
6. Wnt signaling and the VDR: evidence for cancer prevention	245		

## 16. Vitamin D and the kidney

KITTRAWE KKRITMETAPAK, PETER J. TEBBEN, AND  
RAJIV KUMAR

1. Introduction	301
2. Calcium handling by the kidney	302
3. Phosphate handling by the kidney	303
4. Role of the kidney in the metabolism of 25(OH)D	303
5. Effects of vitamin D, 25(OH)D, and 1,25(OH) <sub>2</sub> D on the renal handling of calcium and phosphate	307
6. Distribution and regulation of vitamin D-dependent proteins in the kidney	310
7. Mechanisms of altered vitamin D metabolism in kidney disease	318
8. Conclusion	319
9. Summary points	320
References	320

## 17. Vitamin D and the calcium-sensing receptor

CHIA-LING TU AND WENHAN CHANG

1. Introduction	331
2. Development of the parathyroid glands	332
3. The parathyroid hormone gene	332
4. The calcium-sensing receptor	332
5. Overview of systemic Ca <sup>2+</sup> homeostasis	340
6. Actions of CaSR and VDR in tissues uninvolved in extracellular Ca <sup>2+</sup> homeostasis	350
7. Summary points	352
References	353
Further reading	363

## 18. Regulation of intestinal calcium and phosphate absorption by vitamin D

JAMES C. FLEET, SYLVIA CHRISTAKOS, AND  
HENG JIANG

1. Introduction	365
2. Intestinal calcium absorption	365
3. Molecular models of calcium absorption	368
4. Physiologic regulation of vitamin D-mediated intestinal calcium absorption	371
5. Genomic and genetic studies of vitamin D-regulated intestinal calcium absorption	374
6. Conclusions regarding the role of vitamin D on intestinal calcium absorption	375
7. An overview of intestinal phosphate absorption	375
8. Conclusion regarding the role of vitamin D on phosphate absorption	378
9. Summary points	378
References	378
Further reading	383

## 19. Vitamin D and FGF23

NEJLA LATIC AND REINHOLD G. ERBEN

1. Introduction	385
2. FGF23 signaling	386
3. Role of vitamin D signaling in the regulation of FGF23 production	387
4. Regulation of renal 1,25(OH) <sub>2</sub> D production by FGF23	388
5. Role of FGF23-mediated regulation of vitamin D metabolism in health and disease	390
6. Conclusion	391
7. Summary points	391
References	391

## 20. Osteoclastogenesis and vitamin D

YUKO NAKAMICHI,  
NAOYUKI TAKAHASHI, TATSUO SUDA, AND  
NOBUYUKI UDAGAWA

1. Introduction	395
2. Regulation of mineral metabolism by vitamin D, parathyroid hormone, and fibroblast growth factor 23	396
3. Phenotypes of vitamin D receptor knockout mice	398
4. Osteoclast lineage-specific vitamin D receptor knockout mice	400
5. Regulation of osteoclastogenesis by the receptor activator of nuclear factor- $\kappa$ B ligand/receptor activator of nuclear factor- $\kappa$ B/osteoprotegerin system	400
6. In Vitro regulation of osteoclastogenesis by vitamin D	401
7. In Vivo regulation of osteoclastogenesis by vitamin D	401
8. Conclusion	405
9. Summary points	405
References	406
Further reading	408

## 21. Vitamin D activity in osteoblasts and osteocytes

PAUL H. ANDERSON

1. Introduction	409
2. Properties of osteoblasts and osteocytes	409
3. The local and endocrine effects of 1,25(OH) <sub>2</sub> D <sub>3</sub> on osteoblast and osteocyte function	412
4. Conclusion	415
5. Summary points	415
References	415
Further reading	418

## 22. Cartilage

BARBARA D. BOYAN, NIELS C. ASMUSSEN,  
MARYAM DOROUDI, KAYLA M. SCOTT, AND  
ZVI SCHWARTZ

1. Introduction	419
2. Properties of cartilage tissues	420
3. Regulation by vitamin D	420
4. Conclusion	429
5. Summary points	430
References	430
Further reading	434

## 23. Vitamin D and bone: an integrated approach

LIEVE VERLINDEN AND GEERT CARMELIET

1. Introduction: a link between bone metabolism, calcium homeostasis, and $1,25(\text{OH})_2\text{D}_3$ activity	435
2. $1,25(\text{OH})_2\text{D}_3$ and bone metabolism during a positive calcium balance	436
3. Negative calcium balance resulting from insufficient vitamin D action	441
4. Negative calcium balance with sufficient vitamin D	444
5. Vitamin D and FGF23	446
6. Conclusion	447
7. Summary points	448
References	448
Further reading	452

## 24. Vitamin D in dentoalveolar and oral health

BRIAN L. FOSTER, EMILY Y. CHU, AND  
PHILIPPE P. HUJOEL

1. Introduction	453
2. A brief primer on dentoalveolar cells and tissues	453
3. Vitamin D metabolism and mechanisms of action on dental cells	458
4. Vitamin D, rickets, and the oral tissues	460
5. Hereditary vitamin D-related diseases affecting dentoalveolar tissues	463
6. Other inherited rachitic conditions affecting vitamin D metabolism	467
7. Vitamin D and oral health	471
8. Conclusions	474
9. Summary points	475
Acknowledgments	475
References	475
Further reading	484

## 25. Role of vitamin D in skin and hair biology

DANIEL D. BIKLE AND MARIE B. DEMAY

1. Introduction	485
2. Cutaneous production of vitamin D and its biologically active metabolites	486
3. Regulation of keratinocyte differentiation	488
4. Role of vitamin D signaling in skin cancer	496
5. Role of vitamin D signaling in epidermal wound repair	498
6. Role of vitamin D signaling in hair follicle cycling	498
7. Summary points	502
References	502

## 26. Vitamin D and the cardiovascular system

ADRIANA DUSSO, KEVIN T. BAUERLE, AND  
CARLOS BERNAL-MIZRACHI

1. Introduction	511
2. Vascular physiology	512
3. Identification of the VDR in the vascular system	512
4. Vascular relaxation	513
5. Structural vascular changes	515
6. Vascular inflammation	516
7. Angiogenesis	518
8. Blood coagulation and fibrinolysis	520
9. Animal models	520
10. Clinical studies in cardiovascular disease	524
11. Conclusion	527
12. Summary points	528
References	528
Further reading	535

## 27. Vitamin D, brain development and function

DARRYL EYLES, XIAOYING CUI, AND JOHN J. MCGRATH

1. Introduction	537
2. Vitamin D signaling in the brain	538
3. Dietary vitamin D deficiency, effect on brain development, and function	540
4. Vitamin D regulates essential processes in normal brain development	546
5. Possible neuroprotective mechanisms of vitamin D in the developing brain	549
6. Developmental vitamin D deficiency and neuropsychiatric disorders	551
7. Vitamin D supplementation and neurodevelopmental outcomes	553
8. Conclusions	554
9. Summary points	554
References	555



## 28. Vitamin D, VDR, and adipose tissue: focus on cellular mechanisms

CARMEN J. NARVAEZ AND JOELLEN WELSH

1. Introduction	563
2. Storage of vitamin D metabolites in adipose tissue	564
3. Effects of 1,25(OH) <sub>2</sub> D on adipogenesis at the cellular level	566
4. Effect of 1,25(OH) <sub>2</sub> D on human mesenchymal precursors	571
5. Vitamin D receptor regulation of thermogenesis in cell systems	573
6. Impact of vitamin D signaling on adiposity in mouse models	574
7. Effect of vitamin D deficiency and supplementation on adiposity and metabolism in animal models	577
8. Effect of the vitamin D pathway on inflammation in adipocytes and adipose tissue	579
9. Conclusions	580
10. Summary points	581
References	581

## 29. Vitamin D and skeletal muscle

CHRISTIAN M. GIRGIS

1. Introduction	587
2. Skeletal muscle physiology	587
3. Vitamin D receptor in muscle	589
4. Vitamin D and calcium signaling in muscle	592
5. Vitamin D and phosphate signaling in muscle	593
6. Vitamin D uptake in skeletal muscle	594
7. Vitamin D, insulin signaling, and metabolism in muscle	594
8. Vitamin D, muscle contraction, and strength	595
9. Vitamin D, muscle development, and mass	597
10. Vitamin D, muscle injury, and repair	599
11. Vitamin D and age-related changes in muscle	600
12. Vitamin D and bone muscle crosstalk	601
13. Conclusions	601
14. Summary points	602
References	603
Further reading	607

## 30. Understanding vitamin D from mouse knockout models

DAVID GOLTZMAN,  
ANDREW C. KARAPALIS, RICHARD KREMER, AND  
DENGSHUN MIAO

1. Introduction	609
2. Vitamin D metabolism	609

3. Mineral and skeletal homeostasis	612
4. Calcium transport in intestine and kidney	612
5. Intestinal calcium absorption	613
6. Renal calcium reabsorption	613
7. Intestinal phosphate transport	613
8. Regulation of parathyroid gland function	614
9. Interaction of PTH and 1,25(OH) <sub>2</sub> D on calcium and phosphate homeostasis	614
10. Regulation of FGF23 production	615
11. Anabolic effects of vitamin D on the skeleton: role of VDR and 1 $\alpha$ -OHase in chondrocytes	615
12. Effects on skeletal mineralization	616
13. Effects on bone turnover	616
14. Effects on bone resorption	617
15. Effects on skeletal aging	617
16. Calcitriol and teeth	618
17. Extraskeletal actions of 1,25(OH) <sub>2</sub> D	619
18. Ectopic calcification	619
19. Cancer	619
20. Skin and hair follicles	621
21. Epidermal differentiation and the immune system	622
22. Conclusion	625
23. Summary points	625
References	625
Further reading	632

## 31. Pharmacology and pharmacokinetics of vitamin D

INEZ SCHOENMAKERS AND KERRY S. JONES

1. Overview	633
2. Vitamin D absorption	634
3. Kinetics of cutaneous synthesis	637
4. Tissue distribution	638
5. Metabolism and determinants of metabolic fate of vitamin D	640
6. Half-lives of vitamin D metabolites	642
7. Factors influencing half-life of vitamin D metabolites	644
8. The pharmacokinetics of bound and free vitamin D metabolites	647
9. Influence of DBP genotype and concentration on vitamin D metabolism and 25(OH)D half-life	649
10. Dose-response to vitamin D supplementation	650
11. The 3-epimer of 25(OH)D and 1,25(OH) <sub>2</sub> D	655
12. Catabolism and excretion	656
13. Vitamin D toxicity	656
14. Summary points	658
Acknowledgments	658
References	658

### 32. Vitamin D as a modifier of genomic function and phenotypic expression during pregnancy

CAROL L. WAGNER AND BRUCE W. HOLLIS

1. Introduction	669
2. Defining a “normal” circulating 25(OH)D concentration in humans	670
3. Unique vitamin D metabolism during pregnancy	671
4. Placental transfer of vitamin D to the fetus and its function	671
5. Vitamin D deficiency during pregnancy: Animal models and human studies	671
6. Vitamin D deficiency during pregnancy: Human studies	675
7. Conclusions	686
8. Summary points	686
References	686
Further reading	692

### 33. The role of vitamin D physiology in regulating calcium and bone metabolism in mother and child: pregnancy, lactation, postweaning, fetus, and neonate

BRITTANY A. RYAN AND CHRISTOPHER S. KOVACS

1. Introduction	693
2. Pregnancy	694
3. Lactation and postweaning recovery	705
4. The fetus	716
5. Neonate and infant	731
6. Conclusion	740
7. Summary points	742
References	742

### 34. Vitamin D and the placenta

JANE K. CLEAL, ROHAN M. LEWIS, AND NICHOLAS C. HARVEY

1. Introduction	761
2. The human placenta	762
3. Vitamin D, pregnancy, and the human placenta	765
4. Vitamin D effects on the placenta	769
5. Vitamin D, the placenta, and lifelong health	771
6. Conclusion	773
7. Summary points	773
References	773

### 35. Consequences of perinatal vitamin D deficiency on later bone health

REBECCA J. MOON, ELIZABETH M. CURTIS, JANE K. CLEAL, ELAINE M. DENNISON, CYRUS COOPER, AND NICHOLAS C. HARVEY

1. Introduction	777
2. Vitamin D physiology and epidemiology in pregnancy	777
3. The lifecourse determinants of osteoporosis	778
4. <i>In utero</i> vitamin D deficiency and bone health	784
5. Conclusion	792
6. Summary points	793
Acknowledgments	793
References	793
Further reading	799

### 36. Adolescence and acquisition of peak bone mass

KEVIN D. CASHMAN, RICHARD LEWIS, AND CONNIE M. WEAVER

1. Introduction	801
2. Pubertal bone acquisition	802
3. Serum 25(OH)D in children and adolescents	806
4. Vitamin D and intermediate endpoints of vitamin D and bone metabolism	811
5. Vitamin D and other predictors of calcium retention	815
6. Vitamin D and bone	815
7. Vitamin D and muscle	821
8. Vitamin D requirements in children and adolescents	821
9. Conclusions	823
10. Summary points	823
References	824
Further reading	829

### 37. The role of vitamin D in orthopedic surgery

AASIS UNNANUNTANA, KATHERINE HASELTINE, TEERAPAT TUTAWORN, ALEKSEY DVORZHINSKIY, AND JOSEPH M. LANE

1. Introduction	831
2. Impact on orthopedic trauma	832
3. Impact on pediatric orthopedics	837
4. Impact on total joint arthroplasty	840
5. Conclusions	844
6. Summary points	845
References	845
Further reading	853



38. Vitamin D and muscle performance in athletes and older adults		13. Concluding remarks and future perspectives	917
LISA CEGLIA		14. Summary points	918
1. Introduction	855	References	918
2. Vitamin D—deficient myopathy	856		
3. Vitamin D and athletic performance	856	41. Vitamin D and the renin—angiotensin system	
4. Vitamin D and muscle strength and performance in older adults	860	YAN CHUN LI	
5. Conclusion	867	1. Introduction	925
6. Summary points	867	2. Overview of the renin—angiotensin system	925
References	867	3. Vitamin D regulation of the renin—angiotensin system	930
Further reading	871	4. Vitamin D and the renin—angiotensin system in disease	934
		5. Conclusion	943
39. Role in reproductive biology and dysfunction in women		6. Summary points	943
LUBNA PAL AND HUGH S. TAYLOR		References	944
1. Introduction	873		
2. Relevance of vitamin D for female reproductive biology: experimental models	874	42. Vitamin D: presence and function in the eye	
3. Relevance of vitamin D for female reproductive biology: gynecological disorders	874	GRAHAM R. WALLACE, SAAEHA RAUZ, AND DHRUV PAREKH	
4. Relevance of vitamin D for human female reproductive biology: human data	875	1. Introduction	953
5. Relevance of vitamin D in female reproductive disorders	876	2. Vitamin D and the eye	954
6. Conclusions	883	3. Ocular infection	955
7. Summary points	883	4. Dry eye disease	955
References	884	5. Cataract	958
		6. Glaucoma	959
40. Vitamin D, reproductive endocrinology, and male reproductive organ function in health and disease		7. Uveitis	960
IDA MARIE BOISEN, RUNE HOLT, IREEN KOOIJ, SAM KAFI YAHYAVI, LI JUEL MORTENSEN, AND MARTIN BLOMBERG JENSEN		8. Age-related macular degeneration	962
1. Introduction	889	9. Diabetic retinopathy	965
2. Effects on Leydig cell function and estrogen signaling	893	10. Conclusion	968
3. Vitamin D and sex steroids	896	11. Summary points	968
4. Vitamin D and Sertoli cell function	900	References	968
5. Vitamin D and germ cell function	900	Further reading	973
6. Vitamin D in human sperm	902		
7. Vitamin D and sperm function	903	43. Adult vitamin D deficiency and adverse brain outcomes	
8. Vitamin D, epididymis, prostate, and seminal vesicle	905	PHOEBE MAYNE, DANA BRADFORD, NATALIE J. GROVES, JOHN J. MCGRATH, AND THOMAS H.J. BURNE	
9. Vitamin D and reproductive hormones	908	1. Introduction	975
10. Vitamin D, semen quality, and fertility: Functional models	909	2. Mechanisms of vitamin D action in the brain	976
11. Vitamin D, semen quality, and fertility: Human studies	912	3. Vitamin D and adverse brain outcomes in humans	979
12. Testicular germ cell tumors	916	4. Vitamin D and adverse brain outcomes in animals	985
		5. Conclusions	989
		6. Summary points	989
		References	990
		Further reading	996

## 44. Vitamin D and the lung

KAREN MAES, GHISLAINE GAYAN-RAMIREZ, AND WIM JANSSENS

1. Introduction	997
2. Vitamin D in early life	998
3. Role of vitamin D in the defense against respiratory infections	1000
4. Vitamin D and lung infections	1004
5. COVID-19	1006
6. Conclusion	1007
7. Summary points	1007
References	1007

## 45. Design and synthesis of vitamin D analogs

MIGUEL A. MAESTRO

1. Introduction	1013
2. Synthetic approaches to vitamin D trienic system	1013
3. 19-Nor/21- <i>epi</i> /C-2 substituted analogs of 1,25(OH) <sub>2</sub> D <sub>3</sub>	1014
4. Side chain analogs of 1,25(OH) <sub>2</sub> D <sub>3</sub>	1017
5. A-ring analogs of 1,25(OH) <sub>2</sub> D <sub>3</sub>	1019
6. CD-ring modified analogs of 1,25(OH) <sub>2</sub> D <sub>3</sub>	1020
7. Fluorinated analogs of 1,25(OH) <sub>2</sub> D <sub>3</sub>	1021
8. 14- <i>epi</i> analogs of 1,25(OH) <sub>2</sub> D <sub>3</sub>	1022
9. Non-steroidal compounds	1022
10. Miscellaneous	1022
11. Advances in synthetic methodologies	1024
12. Conclusion	1024
13. Summary points	1025
References	1025

## 46. Structure function relationships of VDR ligands

HIROYUKI KOJIMA AND KEIKO YAMAMOTO

1. Introduction	1027
2. Agonist-bound structures of the VDR-LBD	1030
3. Apo structure of the VDR-LBD	1038
4. Antagonist-bound structures of the VDR-LBD	1040
5. Partial agonist-bound structures of the VDR-LBD	1046
6. Conclusions and perspectives	1047
7. Summary points	1047
References	1047
Further reading	1051

## 47. Modulating vitamin D receptor—coregulator binding with small molecules

DANIEL A. WEBB, MAIJA B. LEE, AND LEGGY A. ARNOLD

1. Introduction	1053
2. VDR coactivators	1054
3. VDR corepressors	1054
4. Peptide-based inhibitors of VDR—coregulator interactions	1055
5. Small molecule—based inhibitors of VDR—coregulator interactions	1056
6. Conclusion	1058
7. Summary points	1058
References	1058
Further reading	1061

## 48. Mass spectrometry assays of vitamin D metabolites

MARTIN KAUFMANN, LUSIA SEPIASHVILI, AND RAVINDER J. SINGH

1. Introduction	1063
2. Approaches and utility of vitamin D metabolite measurements in the clinical laboratory setting	1064
3. Overview of methodology for analysis of circulating vitamin D	1069
4. Application of LC—MS/MS to the study of vitamin D metabolism in animal models	1074
5. Conclusions	1079
6. Summary points	1080
References	1080

## 49. Measurement and quality assessment of total and free 25-hydroxyvitamin D

CHRISTOPHER T. SEMPOS, EMMA L. WILLIAMS, DIANE C. KRUEGER, NEIL C. BINKLEY, AND RENE F. CHUN

1. Introduction section 1: 25-hydroxyvitamin D assay standardization	1085
2. Vitamin D standardization program (VDSP) objectives	1086
3. Introduction to performance testing (PT)/external quality assessment schemes (EQA)	1093
4. Prospective standardization	1094
5. Protocol for retrospective standardization	1096
6. Performance testing/quality assessment of assays for vitamin D metabolites	1099
7. Beyond standardization	1101
8. Vitamin D standardization program efforts—into the future	1101

<b>xiv</b>	Contents	
9. Summary and conclusions	1104	3. Mass spectrometry imaging 1116
10. Section 2: Free and bioavailable 25-hydroxyvitamin D	1104	4. Mass spectrometry imaging methods for spatial distribution of vitamin D metabolites in tissues 1122
11. Summary points	1108	5. Future MSI platforms and technological developments 1125
References	1109	6. Conclusion 1126
50 Vitamin D tissue distribution by mass spectrometry imaging		7. Summary points 1126
DIEGO F. COBICE AND KARL W. SMITH		References 1126
1. Introduction	1115	Further reading 1129
2. Vitamin D	1115	<b>Index for Volume 1 1131</b>

## VOLUME II

### 51. Defining thresholds for vitamin D I: scientific rationale for serum 25-hydroxyvitamin D cutoffs of 25 and 50 nmol/L

ANDREA L. DARLING AND SUSAN A. LANHAM-NEW

1. Introduction 1
2. Justification for serum 25(OH)D concentration >25 nmol/L 1
3. Justification for serum 25(OH)D concentration >50 nmol/L 3
4. Issues with reaching 25(OH)D thresholds for specific groups 5
5. Other issues surrounding definition of thresholds for vitamin D 7
6. Conclusion 7
7. Summary points 7
- References 7

### 52. Defining thresholds for vitamin D II

REINHOLD VIETH

1. Introduction 11
2. Human biology 14
3. Nutrition clinical trials cannot match the quality of evidence achieved with drugs 15
4. Cancer 17
5. Mortality 19
6. Cardiovascular 20
7. Toxicity 21
8. Multiple sclerosis 21
9. COVID-19 22
10. Conclusion 26
11. Summary points 26
- References 26

### 53. Methods of evaluating population studies of vitamin D: strengths and weaknesses

EDWARD GIOVANNUCCI

1. Introduction 31
2. Methods of assessing vitamin D status 32
3. Main challenges in the epidemiologic study of vitamin D and disease 36
4. Summary of study designs of vitamin D 38
5. Conclusions 43
6. Summary points 44
- References 44
- Further reading 46

### 54. Worldwide vitamin D status

NATASJA VAN SCHOOR, RENATE DE JONGH, AND PAUL LIPS

1. Introduction 47
2. Assessment of vitamin D status 48
3. Study selection 49
4. Multicenter and global studies using a central laboratory facility 66
5. Ethnicity/migration 66
6. Nutrition 66
7. Risk groups 66
8. Trends in vitamin D status 69
9. Implications 69
10. Conclusions 70
11. Summary points 70
- References 70

### 55. Vitamin D in food—Compounds, stability, sources

JETTE JAKOBSEN AND MARIE BAGGE JENSEN

1. Introduction 77
2. Chemical structure of vitamin D vitamers 78
3. Stability of vitamin D 78
4. Analytical methodology 81
5. Vitamin D in food 82
6. Conclusion 92
7. Summary points 92
- References 92
- Further reading 96

### 56. Determinants of vitamin D levels from sun exposure: a global perspective

WILLIAM B. GRANT, HARJIT P. BHATTOA, AND PAWEŁ PLUDOWSKI

1. Introduction 97
2. Vitamin D production from solar UVB exposure 97
3. Factors affecting 25-hydroxyvitamin D concentrations 98
4. Endogenous characteristics 101
5. Behavioral determinants 102
6. Predicting vitamin D deficiency 107
7. Nonvitamin D benefits from solar UV exposure 107
8. Conclusion 108
9. Summary points 108
- References 108

### 57. Vitamin D supplement use as a public health strategy to augment diet and sustain population adequacy

SUSAN J. WHITING AND MONA S. CALVO

1. Introduction 115

2. Thresholds for 25(OH)D	117	5. Variants with evidence for replicated association with 25(OH)D	187
3. Country-level guidelines for supplements use	118	6. Gene—environment interaction	192
4. Supplement use improves vitamin D status: Canada as a case study	120	7. Applications and practical implications	193
5. Characteristics of vitamin D supplement users	123	8. Conclusions	194
6. Considerations when choosing to take a vitamin D supplement	124	9. Summary points	194
7. Safety of vitamin D supplements	127	References	195
8. Guidance is needed for use of vitamin D supplements	129	61. Effect of vitamin D on health and disease: evidence from Mendelian randomization	
9. Conclusion	131	KATERINA TRAJANOSKA, FERNANDO RIVADENEIRA, J. BRENT RICHARDS, AND DESPOINA MANOUSAKI	
10. Summary points	131	1. Introduction	201
References	131	2. Type 2 diabetes	213
58. Vitamin D and food fortification		3. Risk of cancers	214
KEVIN D. CASHMAN AND MAIREAD KIELY		4. Risk of cardiovascular events	214
1. Introduction	135	5. Hypertension	215
2. Current intakes of vitamin D in young and adult populations in North America and Europe and how these compare against dietary targets	138	6. Musculoskeletal outcomes	215
3. Dietary strategies for increasing vitamin D intake: bridging the gap by food fortification	143	7. Lung function and respiratory conditions	216
4. Consideration of other vitamin D—fortified foods	148	8. Immune disorders	216
5. Vitamin D—biofortified foods	150	9. Pregnancy-related outcomes	217
6. Safety considerations and prerequisite data required prior to initiating public health measures	154	10. Neuropsychiatric outcomes	217
7. Conclusions	155	11. COVID-19	217
8. Summary points	156	12. Mortality risk and aging	217
References	156	13. Other outcomes	218
Further reading	160	14. Nonlinear MR studies	218
59. Bariatric surgery, vitamin D, and bone loss		15. Conclusion	219
TIFFANY Y. KIM AND ANNE L. SCHAFER		16. Summary points	219
1. Introduction	161	References	219
2. Skeletal effects of bariatric surgery	163	Further reading	222
3. Potential mechanisms of bone loss	166	62. Growth plate histology, bone histomorphometry, and radiologic features of nutritional rickets and osteomalacia	
4. Prevention and treatment of skeletal effects	175	SUMA UDAY AND WOLFGANG HÖGLER	
5. Summary points	177	1. Introduction	223
Acknowledgments	177	2. Terminology related to bone formation and mineralization	224
References	177	3. The growth plate	224
60. Genetic determinants of 25-hydroxyvitamin D concentrations		4. Mineralization	226
ELINA HYPPÖNEN, ANG ZHOU, AND KARANI S. VIMALESWARAN		5. Bone histomorphometry in osteomalacia	227
1. Introduction	185	6. Bone mineral density distribution in osteomalacia	228
2. 25(OH)D synthesis and metabolism	185	7. Radiological features of rickets and osteomalacia	229
3. Heritability of serum 25(OH)D levels	186	8. Radiographic differences between various forms of rickets	231
4. Genome-wide association studies of 25(OH)D	186	9. Imaging choice in rickets and osteomalacia	236
		10. Conclusion	237

11. Summary points	238	8. Hyperphosphatemia-mediated hypocalcemia	314
References	238	9. Hypocalcemia due to accelerated skeletal mineralization	315
Further reading	240	10. Medical illness	315
63. Vitamin D deficiency and nutritional rickets in infants and children		11. Treatment of hypocalcemia	316
JOHN M. PETTIFOR, KEBASHNI THANDRAYEN, AND TOM D. THACHER		12. Conclusion	319
Abbreviations	241	13. Summary points	319
Index terms	241	Acknowledgments	319
1. Introduction	241	References	319
2. Historical perspective	242	Further reading	325
3. The epidemiology of vitamin D deficiency and nutritional rickets	242	66. Vitamin D hydroxylation—deficient rickets, type 1A	
4. Clinical presentation	246	FRANCIS H. GLORIEUX AND RENÉ ST-ARNAUD	
5. Biochemical abnormalities	248	1. Introduction	327
6. Biomarkers of active rickets	249	2. Clinical manifestations	328
7. Radiologic changes	251	3. Biochemical findings	329
8. The growth plate in rickets	252	4. Genetic and molecular studies	329
9. Treatment	252	5. Treatment of VDDR1A	330
10. Prevention	254	6. Evolution of VDDR1A under treatment from childhood to adulthood	332
11. Dietary calcium deficiency	258	7. Conclusion	335
12. Rickets of prematurity or metabolic bone disease of prematurity	263	8. Summary points	336
13. The pathogenetic spectrum of nutritional rickets	265	References	336
14. Conclusion	268	67. The role of genetic variation in CYP2R1, the principal vitamin D 25-hydroxylase, and CYP3A4 in vitamin D homeostasis	
References	269	JEFFREY D. ROIZEN AND MICHAEL A. LEVINE	
Further reading	279	1. Introduction	341
64. Clinical disorders of phosphate homeostasis		2. CYP2R1 is the principal human vitamin D 25-hydroxylase	342
EVA S. LIU AND HARALD JÜPPNER		3. CYP2R1 and vitamin D—dependent rickets	345
1. Introduction	281	4. CYP3A4 and vitamin D—dependent rickets	349
2. Phosphate homeostasis	281	5. In vitro and in silico analyses of CYP2R1 function	352
3. Disorders of phosphate homeostasis	284	6. Associations between CYP2R1 variants and disease	353
4. Conclusion	295	7. Regulation of CYP2R1 in health and disease	354
5. Summary points	295	8. Conclusion	355
References	295	9. Summary points	355
Further reading	302	References	355
65. The hypocalcemic disorders		Further reading	357
NADIA MERCHANT AND RACHEL I. GAFNI		68. Hereditary 1,25-dihydroxyvitamin D resistant rickets (VDDR-2A)	
1. Introduction	303	PETER J. MALLOY, DOV TIOSANO, AND DAVID FELDMAN	
2. Physiology of hypocalcemia	303	1. Introduction	359
3. Clinical manifestations of hypocalcemia	305	2. Clinical features	361
4. Differential diagnosis of hypocalcemia	306	3. Mechanism of 1,25(OH) <sub>2</sub> D action relevant to HVDDR (VDDR-2A)	364
5. Parathyroid hormone—independent hypocalcemia	312		
6. Genetic forms of vitamin D—dependent rickets	313		
7. Dietary calcium deficiency	314		

4. Cellular basis of HVDRR (VDDR-2A)	365	12. Mendelian randomization studies	428
5. Mutations that cause HVDRR (VDDR-2A)	373	13. Conclusions and future directions	428
6. Therapy of HVDRR (VDDR-2) patients	382	14. Summary points	428
7. Hypothesis regarding the mechanism for spontaneous healing: outgrowing the need for substantial calcium supplementation in many HVDRR patients after puberty	385	References	429
8. Alopecia	387		
9. Extra-skeletal actions of vitamin D in HVDRR (VDDR-2A) patients	388	71. Adult vitamin D deficiency—fracture and fall prevention: findings from randomized controlled trials	
10. Conclusions	390	BESS DAWSON-HUGHES AND HEIKE A. BISCHOFF-FERRARI	
11. Summary points	392	1. Introduction	435
References	392	2. Vitamin D and falls	436
		3. Vitamin D and fracture risk	438
69. Infantile hypercalcemia and CYP24A1 mutations		4. Conclusions	440
FILOMENA CETANI, DANIELE CAPELLANI, ALESSANDRO BRANCATELLA, GLENVILLE JONES, AND CLAUDIO MARCOCCI		5. Summary points	441
1. Introduction	401	References	441
2. Historical notes	401	Further reading	442
3. The genetic basis of the disease: molecular genetics of CYP24A1 mutations	402		
4. The pathophysiology of the disease: mutated CYP24A1 in the vitamin D metabolism	403	72. Randomized clinical trials of vitamin D and bone health	
5. Clinical features	404	SHARON H. CHOU AND MERYL S. LEBOFF	
6. Diagnosis	405	1. Introduction: clinical and public health importance	443
7. Therapy	405	2. Findings from systematic reviews and metaanalyses	443
8. Conclusion	407	3. Current recommended intakes based on bone health goals	446
9. Summary points	407	4. RCTs of effects of vitamin D supplementation on bone density	447
References	408	5. RCTs of effects of vitamin D supplementation on fracture risk	448
		6. Implications for clinical practice	451
70. Vitamin D and osteoporosis		7. Conclusion	453
PETER R. EBELING		8. Summary points	453
1. Introduction	411	References	453
2. Effects of vitamin D on the skeleton	412		
3. The role of vitamin D genetic factors in osteoporosis and possible interactions with vitamin D therapy	413	73. Calcifediol as a therapeutic	
4. Seasonal variation in vitamin D status	414	JOHN S. ADAMS, ALBERT SHIEH, AND CHARLES W. BISHOP	
5. Determining optimal serum 25-hydroxy-vitamin D concentrations for musculoskeletal health	415	1. Introduction	457
6. Effects of vitamin D alone or calcium and vitamin D on bone mineral density and falls	416	2. Treatment of vitamin D insufficiency with calcifediol	460
7. Primary fracture prevention with vitamin D or calcium and vitamin D	418	3. Calcifediol treatment of secondary hyperparathyroidism associated with chronic kidney disease	462
8. Metaanalyses	423	4. Calcifediol treatment of microbial disease to boost the human innate and adaptive immune responses	465
9. Safety	425	5. Conclusion	470
10. Effects of active vitamin D analogs on fractures	427	6. Summary points	470
11. Large randomized controlled trials of vitamin D supplementation	427	References	470
		Further reading	474



## 74. Vitamin D and organ transplantation

JOSEPH RAPHAEL, EMILY M. STEIN, AND  
ELIZABETH SHANE

1. Introduction	475
2. Effects of vitamin D on immunity and graft rejection	475
3. Vitamin D deficiency prior to organ transplant	476
4. Vitamin D deficiency following organ transplant	478
5. Treatment of posttransplant bone loss with vitamin D and analogs	480
6. Conclusions	482
7. Summary points	482
References	482

## 75. Vitamin D, obesity, the metabolic syndrome and its sequelae

BARBARA J. BOUCHER AND ELINA HYPÖNEN

1. Introduction	489
2. Adipose tissue as a vitamin D reserve	490
3. Obesity and vitamin D deficiency	491
4. Obesity, DBP, and “free” vitamin D concentrations	492
5. The effect of weight loss on serum 25(OH)D concentrations	493
6. Can vitamin D supplementation prevent, or reduce, obesity?	493
7. Obesity and the efficacy of oral vitamin D supplementation	495
8. Brown adipose tissue, obesity, and vitamin D	496
9. Obesity, vitamin D, and metabolic syndrome	497
10. Importance of early-life vitamin D status for later obesity and subsequent risks of metabolic syndrome, type 2 diabetes mellitus, and cardiovascular disease	501
11. Obesity, vitamin D, and cancer	503
12. Obesity, COVID-19, and vitamin D	503
13. Conclusions	503
14. Summary points	504
References	504
Further reading	510

## 76. The role of vitamin D in type 2 diabetes

ANASTASSIOS G. PITTAS

1. Introduction	511
2. Epidemiology and burden of type 2 diabetes	511
3. Biologic plausibility of the link between vitamin D and type 2 diabetes	512
4. Observational studies on vitamin D and type 2 diabetes	515
5. Vitamin D supplementation in people with normal glucose tolerance or type 2 diabetes	526

6. Vitamin D for prevention of type 2 diabetes in people with prediabetes	533
7. Optimizing blood 25(OH)D levels and the concept of “treat-to-target”	543
8. Effect modification of vitamin D supplementation by BMI	545
9. Safety of vitamin D supplementation for prevention of type 2 diabetes	546
10. Conclusions	546
11. Summary points	547
References	547

## 77. Liver metabolism and disease

BINGNING DONG

1. Introduction	559
2. Vitamin D/VDR signaling and antiinflammatory responses in the liver	561
3. Vitamin D/VDR signaling in liver fibrosis and NASH	562
4. Conclusions	563
5. Summary points	563
References	563

## 78. Vitamin D, hypertension, and cardiovascular disease

ARMIN ZITTERMANN AND STEFAN PILZ

1. Introduction	567
2. Vitamin D metabolism and regulation with special reference to cardiovascular disease	568
3. Vitamin D effects on cardiovascular cells	569
4. Vitamin D effects in experimental animals	569
5. Rickets, osteomalacia, and cardiovascular disease	570
6. Vitamin D status and cardiovascular outcomes in observational studies	570
7. Genetic studies on vitamin D and cardiovascular outcomes	571
8. Vitamin D supplementation studies	574
9. Cardiovascular events in large vitamin D supplementation trials in general populations	577
10. Vitamin D in end-stage organ failure	578
11. Adverse vitamin D effects on the cardiovascular system	579
12. Conclusion	580
13. Summary points	580
References	581
Further reading	586

## 79. Vitamin D and renal disease

ADRIANA DUSSO, KEVIN T. BAUERLE, RONG MEI ZHANG,  
AND CARLOS BERNAL-MIZRACHI

1. Introduction	587
-----------------	-----



2. Renal regulation of the vitamin D endocrine system	588	7. Summary points	641
3. Alterations in vitamin D bioactivation to 25(OH)D in chronic kidney disease	589	References	642
4. Alterations in renal 1,25(OH) <sub>2</sub> D synthesis and 25(OH)D and 1,25(OH) <sub>2</sub> D catabolism in chronic kidney disease	592	Further reading	649
5. Alterations in 1,25(OH) <sub>2</sub> D production by extrarenal 1 $\alpha$ -Hydroxylases in chronic kidney disease	594		
6. Alterations in 1,25(OH) <sub>2</sub> D/VDR endocrine and autocrine/paracrine actions in CKD	596	<b>82. Vitamin D and Paget's disease</b>	
7. Vitamin D control of secondary hyperparathyroidism	599	NORIYOSHI KURIHARA AND G. DAVID ROODMAN	
8. Vitamin D control of skeletal health in CKD unrelated to the attenuation of secondary hyperparathyroidism	602	1. Introduction	651
9. Vitamin D maintenance of renal FGF23/klotho axis: relevance for renal and cardiovascular protection	603	2. Mechanism of action of 1,25(OH) <sub>2</sub> D <sub>3</sub> on osteoclast formation	652
10. Vitamin D control of hypertension and systemic inflammation conferring renal and cardiovascular protection independently of renal or systemic klotho levels	606	3. Increased levels of TAF12 in osteoclast precursors from Paget's disease patients contribute to their hyperresponsivity to 1,25(OH) <sub>2</sub> D <sub>3</sub>	652
11. Progress to improve current recommendations for the evaluation of vitamin D status and for the design of safe and effective vitamin D interventions	609	4. Mechanism of action of TAF12 in the increased 1,25(OH) <sub>2</sub> D <sub>3</sub> responsivity of OCL precursors from Paget's patients	655
12. Summary points	610	5. Marrow stromal cells derived from P62 <sup>P394L</sup> knock-in mice and P62 <sup>P392L</sup> Paget's patients are hyperresponsive to 1,25(OH) <sub>2</sub> D <sub>3</sub> and display enhanced RANKL production in response to 1,25(OH) <sub>2</sub> D <sub>3</sub>	657
References	611	6. Deleting TAF12 in mouse OCLs inhibits bone destruction and bone-remodeling	657
		7. Serum concentration of 1,25(OH) <sub>2</sub> D <sub>3</sub> in Paget's patients	659
<b>80. Vitamin D and kidney stones</b>		8. Conclusions	660
MANSI MEHTA AND DAVID S. GOLDFARB		9. Summary points	660
1. Introduction	619	References	660
2. Calcium kidney stones	619		
3. Vitamin D metabolism	620	<b>83. Vitamin D metabolism and disorders in companion animals</b>	
4. Idiopathic hypercalciuria	620	RICHARD JOHN MELLANBY	
5. Vitamin D supplementation and kidney stone risk	622	1. Introduction	663
6. Conclusions	622	2. Dog	666
7. Summary points	623	3. Cat	670
References	623	4. Conclusions and future directions	671
		5. Summary points	671
<b>81. Hypercalcemia due to vitamin D toxicity</b>		References	672
YASAMAN MOTLAGHZADEH, JOHN P. BILEZIKIAN, AND DEBORAH E. SELLMEYER		Further reading	677
1. Introduction	625		
2. Forms of exogenous vitamin D toxicity	625	<b>84. Overview of vitamin D actions in cancer</b>	
3. Forms of endogenous Vitamin D toxicity	630	MARJOLEIN VAN DRIEL, ALBERTO MUÑOZ, AND JOHANNES P.T.M. VAN LEEUWEN	
4. Mechanisms of vitamin D toxicity	634	1. Introduction	679
5. Clinical manifestations of vitamin D toxicity	638	2. Vitamin D and cancer	679
6. Conclusions	641	3. Vitamin D effects on tumor cells	688
		4. Combination therapy	697
		5. Resistance and vitamin D metabolism	699
		6. Stimulation of proliferation	701
		7. Conclusions	702
		8. Summary points	703
		Acknowledgment	703
		References	703

## 85. Vitamin D status and cancer incidence, mortality, and prognosis

HANSEUL KIM, IRENE SHUI, AND  
EDWARD GIOVANNUCCI

1. Introduction	719
2. Cancer incidence	720
3. Cancer mortality and survival	724
4. Genetic variation and gene expression in the vitamin D pathway	730
5. Conclusions	732
6. Summary points	733
References	733

## 86. Antiproliferative and immunoregulatory actions of vitamin D derivatives on hematological malignancies: control of differentiation, proliferation, and cell death

GEORGE P. STUDZINSKI, ELZBIETA GOCEK, EWA  
MARCINKOWSKA, AND MICHAEL DANILENKO

1. Differentiation of myeloid leukemia cells by vitamin D derivatives	741
2. Signaling and execution of monocytic differentiation induced by VDDs	742
3. Transcription factors in VDD-induced differentiation	752
4. Effects of VDDs on the cell cycle and proliferation of human leukemia cells	755
5. Effects of VDDs on cell survival and cell death	764
6. Effects of VDDs on lymphoid lineage cells and immunity	765
7. Clinical applications of VDD actions against hematopoietic malignancies	768
8. Significance of basic and clinical studies of VDDs in hematopoietic malignancies: from bench to bedside	774
9. Conclusions	775
10. Summary points	775
References	776
Further reading	795

## 87. Vitamin D, inflammation, and cancer

ENIKÖ KALLAY, MARTIN SCHEPELMANN, AND LAURA  
BUBURUZAN

1. Introduction	797
2. Vitamin D and the vitamin D system	798
3. Inflammation	798
4. Inflammation-associated cancer	803
5. Conclusions	813
6. Summary points	814
References	814
Further reading	824

## 88. Vitamin D actions in mammary gland and breast cancer

JOELLEN WELSH AND MIN JI BAK

1. Introduction	825
2. Observational and interventional studies on vitamin D and breast cancer	827
3. Expression and function of the vitamin D pathway in normal and neoplastic breast cells/tissues	832
4. Impact of vitamin D signaling in animal models of breast cancer	839
5. Genomic profiling of VDR agonists in breast cancer model systems	841
6. Mechanistic insight into vitamin D actions in breast cancer	844
7. Conclusion	849
8. Summary points	850
References	850

## 89. Vitamin D and colorectal cancer

GEMMA FERRER-MAYORGA, ALBERTO MUÑOZ, AND  
JOSÉ MANUEL GONZÁLEZ-SANCHO

1. Introduction	859
2. Human observational and intervention studies	863
3. Expression of vitamin D hydroxylases and the vitamin D receptor in colorectal cancer	864
4. Mechanism of action of 1,25(OH) <sub>2</sub> D <sub>3</sub> in colorectal cancer	868
5. Animal models	881
6. Conclusion	885
7. Summary points	886
References	886

## 90. Vitamin D in the management of lung cancer: influence of tumor stage, tumor genotype, and treatment modality

TATIANA SHAUROVA, HANNAH CALKINS, MUKUND  
SESHADRI, CANDACE S. JOHNSON, AND  
PAMELA A. HERSHBERGER

1. Introduction	901
2. Vitamin D may have tumor cell intrinsic and tumor cell extrinsic activities in lung cancer	903
3. The role of vitamin D in the chemoprevention of non-small-cell lung cancer	904
4. The role of vitamin D in management of early-stage lung cancer	905
5. The role of vitamin D in the management of advanced lung cancer	905
6. Strategies to maximize vitamin D signaling in lung cancer	909
7. Conclusions	910
8. Summary points	910

Acknowledgments	911	<b>94. Vitamin D and antibacterial immunity</b>	
References	911	AITEN ISMAILOVA AND JOHN H. WHITE	
Further reading	915		
<b>91. Vitamin D and prostate cancer</b>		1. Introduction	995
LARISA NONN, KIRSTEN KRIEGER, CLAYTON YATES, AND MORAY J. CAMPBELL		2. 1,25(OH) <sub>2</sub> D, barrier integrity, and complement activation	996
1. Introduction: prostate cancer is a heterogenous disease	917	3. 1,25(OH) <sub>2</sub> D induces antimicrobial peptides implicated in antibacterial immunity	997
2. Epidemiologic associations between prostate cancer and vitamin D metabolites	918	4. 1,25(OH) <sub>2</sub> D and autophagy	998
3. The rationale to treat prostate cancer by targeting the VDR	920	5. 1,25(OH) <sub>2</sub> D promotes expression of pattern recognition receptors and cytokines	999
4. High-dimensional data approaches to understanding VDR functions in the prostate	923	6. 1,25(OH) <sub>2</sub> D regulates release of reactive oxygen species	1000
5. Lessons from PCa clinical trials with vitamin D compounds	925	7. Evolutionary aspects of 1,25(OH) <sub>2</sub> D induction of AMPs and PRRs	1000
6. Conclusion	927	8. 1,25(OH) <sub>2</sub> D and neutrophils	1001
7. Summary points	928	9. 1,25(OH) <sub>2</sub> D and natural killer cells	1002
References	928	10. 1,25(OH) <sub>2</sub> D and nonclassical immune cells	1002
Further reading	935	11. In vivo evidence for 1,25(OH) <sub>2</sub> D regulation of antibacterial innate immunity	1002
<b>92. Vitamin D and pancreatic cancer</b>		12. Conclusion	1004
EVANGELINA LÓPEZ DE MATURANA, SLADJANA ZAGORAC, NÚRIA MALATS, AND FRANCISCO X. REAL		13. Summary points	1005
1. Pancreatic cancer: an overview	937	References	1005
2. Pancreatic cancer and vitamin D: epidemiological studies	939	Further reading	1010
3. The biology and genetics of pancreatic cancer	950	<b>95. Vitamin D and antiviral immunity</b>	
4. The biology of vitamin D in pancreatic cancer: experimental studies	954	VANDANA KALIA AND SUROJIT SARKAR	
5. Vitamin D and prognosis in patients with PC	957	1. Introduction	1011
6. Vitamin D intervention studies in patients with PC	958	2. Association between vitamin D insufficiency and viral disease in epidemiological and interventional studies	1012
7. Conclusions	959	3. Immunological mechanisms of viral control	1015
8. Summary points	959	4. Vitamin D and immunomodulation	1015
Acknowledgments	959	5. Innate immune sensing of viruses and 1,25(OH) <sub>2</sub> D	1016
References	959	6. Antimicrobial peptides in antiviral immunity and 1,25(OH) <sub>2</sub> D	1017
<b>93. Sunlight, skin cancer and vitamin D</b>		7. Antiviral cytokine and chemokine responses and 1,25(OH) <sub>2</sub> D	1018
REBECCA S. MASON AND KATIE M. DIXON		8. Natural killer cell responses and 1,25(OH) <sub>2</sub> D	1019
1. Introduction	967	9. Antiviral CD4 T cell immunity and 1,25(OH) <sub>2</sub> D	1019
2. UV-induced DNA damage, repair, and immune suppression	968	10. Antiviral CD8 T cell immunity and 1,25(OH) <sub>2</sub> D	1021
3. The vitamin D system and skin cancers	972	11. B cell responses to viruses and 1,25(OH) <sub>2</sub> D	1021
4. Mechanisms of protection from UV-induced skin tumors	975	12. Conclusions	1022
5. Conclusions	983	13. Summary points	1022
6. Summary points	984	References	1023
References	985	Further reading	1034
Further reading	993		

## 96. Vitamin D and adaptive immunity in health and disease

DREW A. GLENCROSS, CHARLOTTE CHEADLE, AND CATHERINE M. HAWRYLOWICZ

1. Introduction	1035
2. Epidemiological and other evidence supporting a role of vitamin D in immune disease	1036
3. Translational studies as a window to study effects of vitamin D on adaptive immunology	1036
4. Actions of vitamin D on proinflammatory T lymphocyte responses	1037
5. Naïve T cells	1037
6. Effects of vitamin D on CD4+ T cell subsets	1038
7. $\gamma\delta$ T cells	1040
8. CD8+ T cells	1041
9. Actions of vitamin D on regulatory T lymphocyte responses	1041
10. Actions of vitamin D on B lymphocytes	1043
11. Actions of vitamin D on the immune microenvironment and the function of APCs	1043
12. Effects of vitamin D upon immunometabolism in APC and T cells	1044
13. Translational aspects of vitamin D regulation of adaptive immunity—vitamin D and vaccine responses	1045
14. Disparity between association, translational, and clinical studies	1045
15. Protection from exacerbation of airway disease by vitamin D	1048
16. UVB and vitamin D at the skin—how does this compare with oral supplementation	1048
17. Conclusions	1049
18. Summary points	1050
References	1051

## 97. Vitamin D, microbiota, and inflammatory bowel disease

MARGHERITA T. CANTORNA AND JUHI ARORA

1. Introduction	1057
2. What is inflammatory bowel disease?	1057
3. Who gets inflammatory bowel disease?	1058
4. The immune system and inflammatory bowel disease	1062
5. Experimental models of inflammatory bowel disease	1065
6. Current treatments for inflammatory bowel disease	1067
7. Vitamin D as a treatment option for inflammatory bowel disease	1068
8. Conclusions	1069
9. Summary points	1069
References	1069

## 98. Vitamin D and tuberculosis

ADRIAN R. MARTINEAU

1. Introduction	1075
2. The immune response in tuberculosis	1076
3. Influence of vitamin D on the immune response to MTB	1076
4. Historical studies	1079
5. Studies with MTB infection as an end point	1081
6. Studies with active TB disease as end point	1082
7. Studies of treatment outcome	1083
8. Conclusions	1084
9. Summary points	1085
References	1085

## 99. The role of vitamin D in COVID-19

SEBASTIAN T. LUGG AND DAVID R. THICKETT

1. Introduction	1091
2. Vitamin D and immunity and inflammation: relevance to COVID-19 infection	1091
3. Vitamin D and respiratory tract infections	1094
4. Vitamin D and COVID-19: geographical latitude and UV radiation	1094
5. Vitamin D and COVID-19: community observational studies	1095
6. Vitamin D and COVID-19: hospital observational studies	1099
7. Vitamin D and COVID-19: effects of polymorphisms in VDR and vitamin D-binding protein genes	1100
8. Vitamin D and SARS-CoV-2 vaccination response	1100
9. Vitamin D supplementation and COVID-19	1100
10. Current guidance of COVID-19 and vitamin D and future research	1104
11. Conclusions	1104
12. Summary points	1105
References	1105
Further reading	1108

## 100. Vitamin D and type 1 diabetes

PIETER-JAN MARTENS, CONNY GYSEMANS, AND CHANTAL MATHIEU

1. Introduction	1109
2. Effect of vitamin D deficiency on type 1 diabetes presentation	1110
3. Relation between vitamin D-related genes and the genetic predisposition to type 1 diabetes	1110
4. Vitamin D and the beta cell	1111
5. Vitamin D as an immune modulator in type 1 diabetes	1113

6. Interventions using vitamin D to prevent or arrest type 1 diabetes	1113	4. Vitamin D deficiency and RA disease risk, severity, and progression	1190
7. Immune effects of interventions with vitamin D in type 1 diabetes	1116	5. Genetic variation in the vitamin D system and RA	1199
8. Vitamin D analogs and type 1 diabetes	1118	6. Effects of supplementary vitamin D on RA	1200
9. Vitamin D in combination therapy to prevent or arrest type 1 diabetes	1119	7. Conclusions	1201
10. Conclusion	1122	8. Summary points	1201
11. Summary points	1122	References	1201
References	1122	Further reading	1206
 101. Vitamin D mechanisms of protection in multiple sclerosis		 104. Psoriasis and other skin disorders	
COLLEEN E. HAYES, ANNE L. ASTIER, AND MATTHEW R. LINCOLN		JÖRG REICHRATH AND MICHAEL F. HOLICK	
1. Introduction	1130	1. Introduction/historical overview	1207
2. Vitamin D and MS genetics	1131	2. Pathogenesis of psoriasis	1208
3. MS sexual dimorphism	1134	3. The vitamin D system in normal and psoriatic skin	1208
4. CD4 <sup>+</sup> FOXP3 <sup>+</sup> T regulatory cells and hormonal regulation of FOXP3	1137	4. Physiological and pharmacological actions of vitamin D analogs in normal and psoriatic skin	1211
5. Interplay between vitamin D and CD4 <sup>+</sup> type 1 regulatory T cells in MS	1145	5. Clinical use of 1,25(OH) <sub>2</sub> D <sub>3</sub> and its analogs in psoriasis and other skin diseases	1214
6. Oligodendrocytes, DNA methylation, and the methionine cycle	1149	6. Vitamin D in prevention and therapy of other skin diseases	1218
7. Some reflections on vitamin D <sub>3</sub> supplementation trials in MS	1155	7. Evaluation of new vitamin D analogs, with less calcemic activity, which can be used for the treatment of hyperproliferative skin disorders	1223
8. Conclusion	1156	8. Conclusion	1224
9. Summary points	1158	9. Summary points	1224
References	1159	References	1225
 102. Vitamin D and the epidemiology of multiple sclerosis		Further reading	1230
KASSANDRA L. MUNGER AND ALBERTO ASCHERIO		 105. Vitamin D, acute respiratory infection, and Asthma/COPD	
1. Introduction	1167	CARLOS A. CAMARGO, JR	
2. Descriptive epidemiology	1168	1. Introduction	1231
3. Vitamin D and MS—historical perspective	1168	2. Common respiratory disorders	1232
4. Vitamin D and risk of MS	1169	3. Vitamin D and lung development	1235
5. Vitamin D, race, and MS risk	1175	4. Vitamin D and acute respiratory infection	1236
6. Vitamin D and MS disease activity and progression	1175	5. Vitamin D and asthma	1240
7. Pediatric onset MS	1178	6. Vitamin D and Copd	1245
8. Conclusion	1179	7. Potential mechanisms	1248
9. Summary points	1179	8. Future research	1248
References	1179	9. Conclusion	1250
 103. Vitamin D and rheumatoid arthritis		10. Summary points	1251
STEPHANIE HARRISON, ELENA NIKIPHOROU, LOUISA JEFFERY, KARIM RAZA, AND MARTIN HEWISON		Acknowledgments	1251
1. Introduction	1185	References	1251
2. Antiinflammatory effects of vitamin D	1185	 106. Vitamin D and acute illness	
3. Vitamin D and mouse models of RA	1189	KARIN AMREIN AND KENNETH B. CHRISTOPHER	
		1. Introduction	1259
		2. Prevalence of vitamin D deficiency in the critically ill patient	1260

---

Contents		xxv
3. Vitamin D deficiency risk factors in the critically ill	1260	7. Summary points 1272
4. Vitamin D mechanisms in critical illness	1262	References 1272
5. Vitamin D and critical care patients	1265	<b>Index for Volume 2 1281</b>
6. Conclusions	1272	

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# In memoriam

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**Anthony W. Norman, Ph.D. (1938–2019) and  
Helen L. Henry, Ph.D. (1944–2018)**



Anthony (Tony) Norman passed away about a year after the death of his partner in life and science, Helen Henry. Tony was born in Ames, Iowa in 1938 and he grew up in Ann Arbor, Michigan, where his father was a Professor and later on Vice President for Research at the University of Michigan. Tony received his BA degree from Oberlin College in 1959, and then pursued Ph.D. training in Madison, Wisconsin, in the laboratory of Hector F. DeLuca (the laboratory originally founded by Harry Steenbock). Tony was actually the first of a long series of Ph.D. students of Hector. The title of his 1963 Ph.D. thesis was "The preparation, distribution and metabolism of  $^3\text{H}$ -vitamins D<sub>2</sub> and D<sub>3</sub>." He then did his postdoctoral research in 1963–64, in the laboratory of the Nobel Laureate Paul D. Boyer at the University of California, Los Angeles, on the topic of electron transport and oxidative phosphorylation. During this postdoctoral period, Tony started his own research projects at the University of California, Riverside, where he spent his entire academic career. There, his first graduate student was Mark Haussler, who would become another giant in the vitamin D field! The next few years would lead to the discovery that would lead to the discovery that production of the active vitamin D metabolite required mRNA/protein synthesis and that the unknown metabolite

accumulated in the nucleus. Soon thereafter, the active metabolite was identified as  $1,25(\text{OH})_2\text{D}$  in Hector's laboratory as well as in Tony's laboratory and in Egon Kodicek's laboratory (Cambridge, UK). This resulted in a major conceptual milestone: "vitamin D is metabolized into a ligand for a nuclear receptor, VDR, its synthesis is feedback controlled as a hormone and it works as a hormone."

Tony was an innovative and productive scientist and author, as demonstrated by more than 700 peer reviewed publications and an H-index of 98 (2012). His major research topics spanned basic biochemistry to clinical research, and dealt with many aspects of vitamin D metabolism and actions. These topics included calcium-binding proteins, intestinal calcium transport, and the action of vitamin on the immune system. Clinically related publications dealt with the consequences of vitamin D deficiency on glucose metabolism and the therapeutic effects of  $1,25(\text{OH})_2\text{D}$  in patients with chronic renal failure (with J. Coburn). He also had a long-standing fruitful collaboration with Bill Okamura regarding the structure function relationship of vitamin D analogs. Later in his career, he pioneered work on nongenomic or rapid actions of the vitamin D metabolites and analogs.

Tony was not only an original hard-working scientist, but in line with his family history, he was also an avid teacher. His commitment to education was evident not only through his teaching at UC Riverside but also through his role as an author or editor of books on endocrinology (frequently together with Helen Henry) and above all by organizing the serial Vitamin D Workshops. He started this initiative as a small-scale meeting in Frankfurt (Germany) in 1973, followed by a somewhat bigger meeting in Wiesbaden (Germany) and thereafter by holding a meeting every 3 years, alternatively in North America and Europe. These meetings were very well attended (up to more than 600 persons for the 1991 Paris workshop), especially so because the topics included all aspects of vitamin D, from chemistry, basic biochemistry and physiology, to animal research and clinical practice. The focus shifted over time in line with new scientific data and clinical practice. For example, the first meetings paid much



attention to the consequences of chronic renal failure on bone health, whereas at later times, dermatology, cancer, immunology, and osteoporosis were more on the forefront. He also introduced a rule to promote the publication of the proceedings of the meetings, initially in a handbook and later on as a special issue of *The Journal of Steroid Biochemistry and Molecular Biology*, so as to reach out to a larger audience. After the Brugge Meeting in 2009, Tony and his long term coorganizer Roger Bouillon decided to alter the organization of the workshop to allow a rotating group of vitamin D experts the opportunity of developing and managing the workshops every year. Thereby, his legacy of bringing together the vitamin D research community was assured for the future.

Rising to Distinguished Professor of Biochemistry, Tony also played important roles at UC Riverside as Department Chair, Divisional Dean and Program Director, and Chair of the Academic Senate.

Helen Henry was born in 1944 and grew up in Tulsa, Oklahoma. She completed her BSc in 1965 and Ph.D. in 1970 at Washington University in St. Louis. After her postdoctoral research at Ohio State University, she joined Tony's laboratory in Riverside (1974). In 1978, Helen began an independent academic career in the Department of Biochemistry at UC Riverside focused on vitamin D metabolism. Her laboratory made major contributions to the understanding of vitamin D metabolism, particularly regulation of the production of the active vitamin D hormone by the kidney. She pioneered the use of cell culture systems to study renal vitamin D metabolism. With over 80 publications, many of which were coauthored with Tony (and vice versa), Helen had a long and productive career.

Like Tony, Helen had a great passion for teaching and together they developed several courses on nutrition, endocrinology, and introductory biochemistry, which they taught together with other UC Riverside faculty for over 30 years. Even after her retirement in 2008, Helen remained active in teaching and administration at UC Riverside. Both Tony and Helen were also members of the editorial board of several journals, and they jointly edited several editions of the textbook *Hormones*. Evidently, Tony and Helen contributed at least one chapter in each of the editions of Elsevier's *Vitamin D* "bible."

Moreover, Helen and Tony were a team not only in the laboratory but also at home. They were devoted to family and facilitated meetings of their whole family from time to time, often at their "Sea Ranch" in Northern California. Sadly, Helen Henry died, after a long and courageous battle against disease, in May 2018. Tony Norman passed away in June 2019. Tony and Helen are survived by their children Thea, Jacqueline, and Derek and nine grandchildren. There is no doubt

that Tony and Helen left a lasting heritage and will remain remembered and respected by the vitamin D research community.

**Roger Bouillon, MD**  
*Katholieke Universiteit Leuven, Leuven*

**David Goltzman, MD**  
*McGill University, Montreal*

**JoEllen Welsh, PhD**  
*University at Albany Cancer Research Center,  
New York*

**Ronald L. Horst, Ph.D. (1949–2019)**



The field of vitamin D and calcium metabolism lost a beloved colleague on May 23, 2019, when Dr. Ronald L. Horst at the age of 69 passed following a 15-year battle with prostate cancer. He was diagnosed in December of 2004 and was given only about 2 years to survive, but Ron would not accept that diagnosis and fought it for more than a decade and a half. Much of that fight involved the use of large oral doses of vitamin D. When he was first diagnosed back in 2004, ironically, he was quite vitamin D deficient and he quickly corrected that by taking 8000 IU/day of vitamin D. Many of his academic colleagues were horrified by this and warned him of the impending renal damage if he continued and of course Ron ignored them and increased his intake as time went on to 30,000 IU/day. Ron also received traditional therapy for his cancer while taking the vitamin D and his doctors at the Mayo Clinic were constantly amazed by the lack of progression of his cancer. He would tell them that it was the vitamin D providing the protection to which they would respond, we don't think so but keep doing it. It was a gallant fight, but since nobody is ever cured of metastatic prostate cancer, Ron finally succumbed in 2019.

Ron grew up on a dairy farm in West Virginia where he learned the value of hard work that would continue to serve him for the rest of his life. Ron would often tell me that after milking the cows at 4 a.m. he would go to school to rest. Upon graduating from Hedgesville High School in 1967, he began his undergraduate degree at West Virginia University, where he majored of course in Dairy Science and played football for The Mountaineers for a year under Bobby Bowden. Following his graduation from WVU, he went on to the University of Wisconsin–Madison to pursue a Ph.D. in Biochemistry and Dairy Science. During his time in Madison, Ron became involved with the laboratory of Hector DeLuca where he became immersed in the metabolism and measurement of vitamin D and its metabolites. He subsequently applied these techniques to his studies on circulating  $1,25(\text{OH})_2\text{D}$  and bovine milk fever publishing a seminal article in the journal *Science* which was quite a feat for any student.

Ron left his postdoc position with Drs. DeLuca and Neil Jorgensen at UW to begin his career as a scientist at the National Animal Disease Center, USDA ARS in 1977. His mission was to work on metabolic mineral diseases of livestock. His main focus was on milk fever in dairy cows, a disease affecting about 7% of cows annually. To better understand calcium homeostasis in the cow, Ron established improved assays for vitamin D metabolites in blood and tissues. This led to breakthrough understanding of the metabolism of vitamin D compounds within the body. Ron was not satisfied only dealing with his role in agriculture, so he expanded his studies into human medicine through collaboration with too many individuals to list in this memorial. If you search out his PubMed bio, you will see just how extensive this list was. These studies with medical researchers throughout the world enhanced our understanding of renal failure, rickets, hyperparathyroidism, hypercalcemia of malignancy, the association of vitamin D insufficiency and increased risk of developing certain cancers, and osteoporosis. Ron found that vitamin D<sub>3</sub> and its metabolites were more biologically active than were their vitamin D<sub>2</sub> counterparts. His NADC group discovered VDR of bone and intestine declined with age. The discovery of VDR in thymus tissue opened the door to exploration of the role of vitamin D in immune competency. The NADC group Ron led for 29 years is known throughout the world for their work on bovine milk fever; they discovered that high dietary potassium induced a metabolic alkalosis in pregnant dairy cows which reduced the sensitivity of the tissues to PTH. Using this knowledge, Ron and his colleagues devised dietary treatments that decreased the incidence of milk fever in dairy cattle. Ron also elucidated the processes of activation and deactivation of vitamins A and D. The awards that Ron received for his research are truly

astounding and include the American Feed Manufacturing Association Award for Dairy Science, the UpJohn Physiology Award, the Agway Young Scientist Award, the Dean Food Award, the Presidential Meritorious Senior Professional Award, the ARS Scientific Hall of Fame Award, and the Spencer Award in Chemistry. Ron retired from the USDA in 2007 and he and I founded Heartland Assays that analyzes serum and food vitamin D content for commercial entities.

Above all else, Ron was a family man and the best friend a person could ever have. While in Wisconsin, he met the love of his life, Marina 'Myke' Fitzsimmons and they married in 1973. They were blessed with three daughters, Jamie, Emily, and Anna. Those daughters provided Ron with four grandsons who Ron spent as much time with as possible. Following Ron's retirement, he and I purchased homes in Victor, Idaho, where our families spent the summers together until his passing. I can't express the good times we had out there doing outdoor activities including fishing for trout on Henry's Lake. Doing that you could not find a happier man and I miss him every single day.

**Bruce W. Hollis**

*Medical University of South Carolina, Charleston*

#### **Graham Carter (1940–2022)**



On May 18, 2022, we lost Graham Carter, one of the foremost vitamin D analysts and the founder of DEQAS (Vitamin D External Quality Assessment Scheme). Graham established DEQAS in 1989 after a number of international studies brought attention to the poor performance of clinical assays for  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$ . As a Senior Biochemist working at London's

Charing Cross Hospital, Graham recognized that clinical services, commercially available kits, and research assays needed to perform at an acceptable standard and that establishing a scheme to prepare and distribute quarterly samples and then collect the results for external standardization would greatly benefit the vitamin D field. Vitamin D scientists soon embraced the idea and DEQAS had over 1200 participants globally in 2013. Graham's career started with an MSc in Clinical Biochemistry from the University of Surrey; and he was appointed Consultant Clinical Scientist in 1991 and later an Honorary Senior Research Fellow of Imperial College, London, in 2001. Graham continued at the helm of DEQAS uninterrupted from 1989 to 2018.

The DEQAS program has encouraged an improvement in performance by helping clinical chemists and kit manufacturers alike to quickly recognize problems and rectify them. Graham initiated an Advisory Panel in 1997 consisting of vitamin D analysts and steroid biochemists to help with specific problems involving the chemistry of vitamin D and issues connected with data management. Graham Carter carried out this liaison with all parties involved in D assays with considerable diplomacy, recognizing that gradual improvement in the assays was the main goal. Graham served DEQAS faithfully for close to 30 years and among his final achievements was to negotiate an agreement with NIST and later CDC-Atlanta to provide 25(OH)D target values for all samples circulated quarterly. He successfully passed on the reins of DEQAS to the current administrator, Dr. Emma Walker, in 2018 when he retired. Current DEQAS results provide values for the main circulating form 25(OH)D, the hormone 1,25(OH)<sub>2</sub>D, the chief catabolite, 24,25(OH)<sub>2</sub>D, and the neonatal metabolite, 3-epi-25(OH)D. Graham's legacy is the achievement of a truly global organization that has facilitated a big improvement in clinical vitamin D assays and accordingly, scientific publications in the vitamin D field. Graham Carter's death will leave a big void in the field of vitamin D analysis that will be hard to replace.

Graham enjoyed his time away from the laboratory with travel, most significantly to the Caribbean island of Grenada. Having been introduced to the island by his friend and colleague Vernon Scoon, he and his wife Brigitte bought a plot of land and went on to build a home where they lived for many years. When not abroad, his interests were much more local to home in England where he enjoyed ringing church bells at his local church, Ash-cum-Wrotham in Kent. He was also a lifelong musician, and as a child he was the head chorister at Truro Cathedral in Cornwall, where he learned the piano. After a long break, he returned to the piano later in life, ultimately achieving an ABRSM Grade 8 with distinction. He disliked performing to

others, but enjoyed attending concerts and learning and practicing Beethoven piano sonatas. During the pandemic, he found the confinement of lockdown hard, and took up piano lessons by Zoom, a habit he kept up even as his health started to decline with his last piano lesson only a few weeks before his death. Graham is survived by his wife Brigitte and his two sons, Richard and Tim.

*Glenville Jones with input from Hugh Makin, Elaine Gunter (all of DEQAS), and Graham's son Richard Carter*

#### Professor Judith Elizabeth Adams (1945–2017)



Professor Judith Elizabeth Adams, 'Judy' to her friends, was an eminent skeletal radiologist who passed away after a short illness on 30th September 2017. Judy was born 16th May 1945, in Liverpool and grew up in Northern Rhodesia (now Zambia). She trained at University College Hospital, London, UK, with her radiology career beginning in 1972. Her mentors included Sir Godfrey Hounsfield, the inventor of X-ray computed tomography, and Sir Charles Dent, a pioneer of metabolic bone disease and particularly vitamin D metabolism in the United Kingdom. She often reflected on her learnings from both pioneers and undoubtedly, Charles Dent, and latterly Professors Bill Stanbury and Barbara Mawer, enthused her with a passion for vitamin D research. She joined the University of Manchester in 1976 and became a Professor of Radiology and Head of Clinical Radiology in 1993. She served as the Dean, member of council, and Vice President of the Royal College of Radiologists. Judy was a member of many societies, including the



American Society for Bone and Mineral Research, the European Calcified Tissue Society, the Royal Osteoporosis Society and the Bone Research Society (UK), and also the International Skeletal Society and European Society for Skeletal Radiologists. She traveled the world attending the International Bone Densitometry Workshop's and hosting the 1987 Workshop in the United Kingdom.

Judy was a pioneer in the use of radiographic and bone densitometry in the diagnosis of osteoporosis and vertebral fracture recognition. She led and coauthored several national and international guidance documents for the clinical application of bone densitometry in pediatric and adult fields. She played a pivotal role in the collection of one of the first and largest UK reference datasets in children, which has transformed pediatric practice in the United Kingdom. She championed the clinical application of quantitative computed tomography in adults and children. She was a champion of the International Osteoporosis Foundation Vertebral Fracture initiative. Through her role in the European Society for Skeletal Radiology, Judy provided a bridge between the radiology and bone fields.

In collaboration with her colleagues in Manchester, Judy conducted research on vitamin D research studies that evaluated the effects of vitamin D and its active metabolite (1,25-dihydroxyvitamin D) on bone mass, bone geometry, and muscle function across the life course in different populations, particularly the South Asian population of the North-West United Kingdom. Judy also collaborated on studies evaluating the role of vitamin D on muscle function and sarcopenia in adults and children. Her mobile densitometry unit of which she was extremely proud facilitated much of this work across Greater Manchester, UK. She was also involved in research studies and intervention trials of vitamin D in chronic illnesses including cystic fibrosis, chronic renal failure, and coeliac disease. She also used her CT imaging expertise to study spinal canal stenosis and cord compression and thickening of the petrous bone, resulting in deafness in adults with chronic osteomalacia secondary to X-linked hypophosphatemia. In summary, Judy's contributions to vitamin D research have helped advance our understanding of the importance of this nutrient in various populations and chronic health conditions.

The UK Bone Research Society honored Judy by giving her the Dent Award in 2015, in recognition of her contribution to clinical imaging. In 2016, she was awarded the Linda Edwards Award from the Royal Osteoporosis Society, UK, for her outstanding contribution to the Society and to the field of osteoporosis. For radiology, she was awarded the Gold Medal from both the Royal College of Radiologists (2016) and the International Skeletal Society (2007).

Judy was a warm, thoughtful, and loyal friend. She was a great mentor to both of us and many other clinicians and scientists who are indebted to her mentorship and tutelage. Her collaborations spanned the globe. She will be fondly remembered by all for her elegance, her smile and her laugh, her bright clothes, and her endless energy and enthusiasm. Judy was wonderful company both professionally and personally. Outside of work, she loved culture, from Manchester United through to opera, flowers, gardening, and travel. Sadly Judy's husband of 45 years, Professor Peter Adams, an Emeritus Professor of Medicine at the University of Manchester, passed away a week later. They are survived by their two sons, Charles and James, and their grandchildren on whom they doted.

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#### Heide S. Cross (1942–2021)



In 2021, we lost Heide S. Cross, one of the pioneers in vitamin D research, whose goal was to understand the role of vitamin D in cancer, with a main focus on colorectal cancer. Inspired by reports from C. F. Garland and his colleagues—that the risk of colorectal cancer significantly correlates with low vitamin D status as well as nutritional calcium deficit—Heide initiated a

series of studies to elucidate the cellular and molecular mechanisms by which vitamin D—through its hormonally active metabolite 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>)—and calcium could inhibit cell growth and promote differentiation in human colon cancer cells.

Heide identified the key elements in colon tumor cell-specific antiproliferative signaling from both the 1,25(OH)<sub>2</sub>D<sub>3</sub>-activated vitamin D receptor (VDR) as well as from the extracellular calcium-sensing receptor (CaSR), providing for the first time a valid molecular explanation for the well-known preventive effect of calcium in combination with vitamin D against the development of colon tumors. Heide and her group were also the first who discovered that human colon carcinoma cells are an important site of extrarenal expression of the CYP27B1-encoded enzyme 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase, and therefore are able to produce 1,25(OH)<sub>2</sub>D<sub>3</sub> from circulating 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>). She has shown that calcium influences vitamin D metabolism in a cell-specific way, which is highly relevant for the antiproliferative effect of the mineral on colon epithelial cells. Unlike in renal cells, where calcium upregulates expression of the 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), in colonocytes, the mineral ion suppresses expression and activity of the enzyme. Therefore, adequate calcium nutrition must be seen as an important means to counteract catabolism of 1,25(OH)<sub>2</sub>D<sub>3</sub> to maintain the intracellular concentrations of the steroid hormone at a level sufficiently high for effective control of cell growth.

The important finding that expression of CYP27B1 as well as of the VDR rises in parallel with tumor

progression through the adenoma/carcinoma sequence led her to formulate the theory that human colon tumor cells can activate intracellular signaling from the 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR complex as an intrinsic defense system against mitogenic stimuli of all sorts. This concept was shown to be valid also for other human malignancies such as breast and prostate cancer. Through the work of Heide, it has become possible to provide a molecular and cellular explanation for the numerous observations from epidemiological and clinical studies suggesting that deficiency and insufficiency of both vitamin D and calcium is a major risk factor for colorectal cancer and other malignancies.

In addition to her highly recognized scientific achievements, Heide was a teacher and mentor for numerous young scientists at the Medical University of Vienna. It was very important to her that her students gain experience abroad, which she made possible through her many collaborators at renowned institutes such as Johns Hopkins University, Cornell University, Brown University, and Harvard University.

In her private life, Heide was very interested in art and culture. She loved ballet and took dancing lessons after retiring, enjoying the freedom to follow her interests. Shortly after her retirement, Heide had enrolled to study the history of religion at the University of Vienna, in order to, as she said, “understand the world better.” She loved nature and traveling, and spent ever longer periods on the beautiful island of Karpáthos (Greece) every year. We shall miss her dearly.

*Enikő Kallay with input from Meinrad Peterlik and Heide's sister, Helga Lesigang*

# Preface to the fifth edition

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The fifth edition of this textbook on vitamin D represents a landmark in that it is the first edition to be published in the “second century” of vitamin D. 2022 represents the generally accepted 100th anniversary of the first formal reference to vitamin D by Elmer McCollum. The editorial team and I hope that this new edition provides a comprehensive statement on the many advances that have occurred over 100 years of vitamin D research. It covers the fundamental information essential to our understanding of vitamin D—the photobiology that underpins synthesis of vitamin D, the metabolic pathways that enable generation of active metabolites of vitamin D and, of course, the skeletal disorders of vitamin D deficiency that sparked its discovery in 1922. These core features remain at the heart of the Vitamin D book, but are enhanced by new images, references and, in some cases, dramatic new developments such as alternative pathways that generate novel vitamin D metabolites with diverse biological properties ([Chapter 6, \*Alternative Pathways for Vitamin D Metabolism\*](#)). However, “Vitamin D” is much more than a core reference work and the editorial team (Roger Bouillon, Ed Giovannucci, David Goltzman, Mark Myer and JoEllen Welsh) and I have sought to present the ever widening impact of vitamin D on human (and non-human in the case of [Chapter 83, \*Vitamin D and Companion Animals\*](#)) health. In the 5 years since publication of the previous edition, the vitamin D research landscape has changed dramatically. This is to be expected for almost any facet of biomedical science, and research in general welcomes change. The vitamin D research field has embraced emerging technologies such as single cell OMICs and mass spectrometry imaging that have provided new perspectives on the mechanism of action of vitamin D. The expanding role of vitamin D in overall health and wellness has spurred multiple large supplementation trials, and the newest trials have been designed to build on lessons learned from earlier studies. The combined effect is a much clearer picture of how vitamin D functions and the most likely health benefits of vitamin D supplementation. In [Chapter 1 \(\*Historical Overview\*\)](#), the previous editors of “Vitamin D”, David Feldman and Wesley Pike provide a synopsis of the many new developments in the field, and which chapters detail these findings within each volume.

When considering the new developments in vitamin D biology, one obvious event that has been very prominent is the COVID-19 pandemic. Beyond the huge impact on healthcare and economies around the globe, COVID-19 brought substantial challenges to research that continue to be felt today. However, the COVID-19 pandemic had a particular resonance with vitamin D research. Very early in the pandemic, it was recognized that those individuals at greatest risk of infection and mortality from COVID-19 were in populations known to experience a high prevalence of vitamin D deficiency. Consequently, the pandemic generated a renewed and broader interest in the immunomodulatory properties of vitamin D and an explosion of publications on vitamin D status and COVID-19. The potential benefits of vitamin D for immune health in general and COVID-19 in particular remain unclear, in part because of the rapid implementation of vaccination programs at the end of 2020. Nevertheless, this new edition of “Vitamin D” reflects the changing landscape on vitamin D and infectious disease, with more chapters on various facets of immunity—notably dedicated chapters for *Vitamin D and Antiviral Immunity* ([Chapter 95](#)) and *Vitamin D and COVID-19* ([Chapter 99](#)).

Although the 5 years since the last edition of this book have witnessed a dramatic expansion of research on and public awareness of vitamin D, the passage of time has also seen the loss of several prominent vitamin D researchers. The deaths of six giants of vitamin D research—Judy Adams, Graham Carter, Heide Cross, Helen Henry, Ron Horst, and Tony Norman—are commemorated in dedicated memorials at the beginning of each volume of the book. The world of vitamin D is poorer without them. Recent years have also seen the passing of Roxanne Hall who was a fundamental part of the vitamin D world in her role as a meeting coordinator for the Vitamin D Workshop from 2012 to 2019. For both junior and established vitamin D researchers, including many authors of chapters in this book, Roxanne was a key facilitator for the annual conference and an integral part of the vitamin D community. In particular, she was the go-to person for all early career vitamin D researchers! Roxanne will be greatly missed. On a personal note, the beginning of 2023 brought the sad news of the

death of Louisa Jeffery a talented vitamin D researcher at Birmingham who worked with myself, Karim Raza and David Sansom. Louisa was younger and less well known than those remembered in the “In Memoriam” section. Nevertheless, she was a devoted vitamin D researcher who published 13 papers on vitamin D and immune function, including three seminal studies on T cell function, and she played a pivotal role in a new chapter on *Vitamin D and Rheumatoid Arthritis* (Chapter 103).

Completing this new edition of “Vitamin D” has involved a considerable effort by authors and editors under very difficult circumstances. Most of the chapters

were commissioned during periods of pandemic lockdown when contributors had conflicting demands and many other things on their minds. I salute everyone who has given their time and effort so generously over the 2 years, it has taken to complete the 106 chapters. I hope that “Vitamin D” will provide inspiration to a new generation of researchers, and that it will also serve to illustrate that the vitamin D research community is alive and well 100 years after its birth.

**Martin Hewison, PhD, Editor-in-Chief**



# List of abbreviations

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1,25(OH) <sub>2</sub> D <sub>3</sub>	1 $\alpha$ ,25-Dihydroxyvitamin D <sub>3</sub>	ATP	Adenosine triphosphate
1,25(OH) <sub>2</sub> D	1 $\alpha$ ,25-Dihydroxyvitamin D	ATRA	All- <i>trans</i> -retinoic acid
1 $\alpha$ -(OH)D <sub>3</sub>	1 $\alpha$ -Hydroxyvitamin D <sub>3</sub>	AUC	Area under the curve
20(OH)D <sub>3</sub>	20-hydroxyvitamin D <sub>3</sub>	BARE	Bile acid response element
24(OH)L <sub>3</sub>	24-hydroxylumisterol <sub>3</sub>	Bax	BCL-2 associated X protein
24,25(OH) <sub>2</sub> D <sub>3</sub>	24,25-Dihydroxyvitamin D <sub>3</sub>	BB	Biobreeding
24,25(OH) <sub>2</sub> D	24,25-Dihydroxyvitamin D	BCa	Breast cancer
25(OH)D <sub>3</sub>	25-Hydroxyvitamin D <sub>3</sub>	BCC	Basal cell carcinoma
25(OH)D	25-Hydroxyvitamin D	Bcl-2	B cell CLL/Lymphoma 2
5-ASA	5-Aminosalicylic acid	Bcl-xl	B cell lymphoma-extra large
7-DHC	7-Dehydrocholesterol	BECN1	Beclin 1
9- <i>cis</i> -RA	9- <i>cis</i> -retinoic acid	BER	Base excision repair
[Ca <sup>2+</sup> ]	Internal calcium ion molar concentration	bFGF	Basic fibroblast growth factor
AA	Arachidonic acid	BFU	Burst-forming unit
AC	Adenylyl cyclase	BGP	Bone Gla protein (osteocalcin)
ACE	Angiotensin-converting enzyme	BHMT	Betaine-homocysteine methyltransferase
ACF	Activation frequency	BLM	Basal lateral membrane
ACTH	Adrenocorticotropin	B <sub>max</sub>	Maximum number of binding sites
ADH	Antidiuretic hormone (vasopressin)	BMC	Bone mineral content
ADHR	Autosomal dominant hypophosphatemic rickets	BMD	Bone mineral density
ADP	Adenosine diphosphate	BMI	Body mass index
AHO	Albright's hereditary osteodystrophy	BMP	Bone morphogenetic protein
AI	Adequate intake	BMU	Basic multicellular unit
AIDS	Acquired immunodeficiency syndrome	bp	Base pairs
Aj.AR	Adjusted apposition rate	BPH	Benign prostatic hyperplasia
Akt	RAC- $\alpha$ serine/threonine-protein kinase	BRAF	v-Raf murine sarcoma viral oncogene homolog B
AKT	Acutely transforming retrovirus AKT8 in rodent T-cell lymphoma	BRD	Bromodomain containing
ALK	Anaplastic lymphoma kinase	BSA	Bovine serum albumin
ALP	Alkaline phosphatase	BUA	Bone ultrasound attenuation
Alum	Aluminum hydroxide	c-MET	Mesenchymal-epithelial transition factor
ANG II	Angiotensin II	CaBP	Calcium-binding protein
ANP	Atrial natriuretic peptide	CAD	Coronary artery disease
AOM	Azoxymethane	CaM	Calmodulin
AP	Apurinic/aprimidinic	CAMP	Cathelicidin antimicrobial peptide
APC	Antigen-presenting cell	cAMP	Cyclic AMP
APD	Aminohydroxypropylidene bisphosphonate	CaSR or CaR	Calcium-sensing receptor
APL	Atrichia with papular lesions	CAT	Chloramphenicol acetyltransferase
AR	Androgen receptor	CBG	Corticosteroid-binding globulin
ARC	Activator-recruited cofactor	CBP	Competitive protein-binding assay
ARE	Androgen responsive element	CC	Chief complaint
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing	CCL2	C-C Motif Chemokine Ligand 2
ATF-4	Activating transcription factor 4	CCL5	C-C Motif Chemokine Ligand 5
ATG5	Autophagy-related 5	CD	Crohn's disease
ATM	DNA strand sensor protein kinase	CDCA	Chenodeoxycholic acid
		CDH1	E-cadherin
		CDK or Cdk	Cyclin-dependent kinase

cDNA	Complementary DNA	DBP	Diastolic blood pressure
CDP	Collagenase-digestible protein	DBP	Vitamin-D-binding protein
Cdx-2	Caudal-related homeodomain protein	DC	Dendritic cell
CEBP	CCAAT enhancer binding protein	DCA	Deoxycholic acid
CFU	Colony-forming unit	DCIS	Ductal carcinomas in situ
cGMP	Cyclic GMP	DCT	Distal convoluted tubule
CGRP	Calcitonin gene-related peptide	DDB1	XPE-binding factor
CHB	Chronic hepatitis B	DDB2	DNA Damage Binding protein 2
CHC	Chronic hepatitis C	DEK	DEK proto-oncogene
CHF	Congestive heart failure	DEQAS	Vitamin D external quality assessment scheme
ChIA-PET	Chromatin interaction analysis by paired-end tag sequencing	DEXA or DXA	Dual energy X-ray absorptiometry
ChIP-seq	Chromatin immunoprecipitation and sequencing	DHEA	Dehydroepiandrosterone
ChIP	Chromatin immunoprecipitation	DHT	Dihydrotachysterol or dihydrotestosterone
CHOP	C/EBP homologous protein	DIC	Disseminated intravascular coagulation
CK-II	Casein kinase II	DIDS	Disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS)
CK1	Casein kinase 1	DIKKOPF-1	Dickkopf-related protein 1
CLIA	Competitive chemiluminescence immunoassay	DIPP	Type 1 Diabetes Prediction and Prevention study
Cm. Ln.	Cement line	DMR	Differentially methylated region
cM	Centimorgans	DMSO	Dimethyl sulfoxide
CMM	Cutaneous malignant melanoma	DNase-seq	DNase I hypersensitivity sequencing
CMV	Cytomegalovirus	DNMT	DNA-methyltransferase
CNS1	Conserved noncoding sequence-1	DR	Direct repeat
CNS	Central nervous system	DRIP	Vitamin D receptor interacting protein
CoA	Co-activator	DSP	Dental sialoprotein
CoR	Co-repressor	DSS	Dextran sodium sulfate
COX	Cyclooxygenase	E <sub>1</sub>	Estrone
CP	Chronic pancreatitis	E <sub>2</sub>	Estradiol
CPBA	Competitive protein-binding assay	EAE	Experimental autoimmune encephalitis
CPD	Cyclobutane pyrimidine dimers	EBT	Electron beam computed technology
cpm	Counts per minute	EBV	Epstein–Barr virus
CRE	cAMP response element	EC <sub>50</sub> or ED <sub>50</sub>	Effective concentration (dose) to cause 50% effect
CREB	cAMP response element binding protein	EC	Endothelial cells
CRF	Chronic renal failure	ECaC	Epithelium calcium channel
CsA	Cyclosporin A	ECF	Extracellular fluid
CSF	Colony-stimulating factor	EDTA	Ethylenediaminetetraacetic acid
CT	Calcitonin or computerized tomography	EFTUD1	Elongation factor like GTPase 1
CTCF	CCCTC-binding factor	EGF	Epidermal growth factor
CTLA-4	Cytotoxic T-lymphocyte antigen 4	EGFR	Epidermal growth factor receptor
CTR	Calcitonin receptor	ELISA	Enzyme-linked immunosorbent assay
CTX	Cerebrotendinous xanthomatosis	EMSA	Electrophoretic mobility shift assay
CTX	Cyclophosphamide	EMT	Epithelial-mesenchymal transition
CVC	Calcifying vascular cell	ENCODE	Encyclopedia of DNA elements
CXCL10	C-X-C motif chemokine ligand 10	EP <sub>1</sub>	PG receptor-1
CYP24A1	Cytochrome P450, 24-hydroxylase	ER	Endoplasmic reticulum
CYP27A1	Cytochrome P450, 25-hydroxylase	ER	Estrogen receptor or endoplasmic reticulum
CYP27B1	Cytochrome P450, 1 $\alpha$ -hydroxylase	ERBB2	erb-b2 receptor tyrosine kinase 2
CYP2R1	Cytochrome P450, 25-hydroxylase	ERE	Estrogen response element
CYP	Cytochrome P450	ERK	Extracellular signal-regulated kinase
DAG	Diacylglycerol	ERp57	Endoplasmic reticulum protein 57
DAISY	Diabetes AutoImmunity Study in the Young	ER $\alpha$	Estrogen receptor alpha
DBD	DNA-binding domain	Et	Endothelin

F	Tumor necrosis factor	HGF	Hepatic growth factor
FACS	Fluorescence-activated cell sorting or sorter	Hh	Hedgehog
FAD	Flavin adenine dinucleotide	HHRH	Hereditary hypophosphatemic rickets with hypercalciuria
FAIRE-seq	Formaldehyde-assisted isolation of regulatory elements sequencing	HIF-1	Hypoxia-inducible factor 1
FCS	Fetal calf serum	HIV	Human immunodeficiency virus
FDA	US Food and Drug Administration	HL	1 $\beta$ ,25- dihydroxyvitamin D <sub>3</sub>
FFA	Free fatty acid	HLA-DR	Human leukocyte antigen DR
FIT	Fracture Intervention Trial	HLA	Human leukocyte antigen
FMTc	Familial medullary thyroid carcinoma	HNF	Hepatocyte nuclear factor
FOXA1	Forkhead box A1	HOMER	Hypergeometric Optimization of Motif EnRichment
FoxP3	Forkhead box P3	HPI	History of present illness
FP	Formation period	HPLC	High-performance liquid chromatography
FRAP	Fluorescence recovery after photobleaching	HPV	Human papilloma virus
FS	Fanconi syndrome	HR	Hairless
FSK	Forskolin	HRE	Hormone response element
FXR	Farnesoid X receptor	HSA	Human serum albumin
FZD	Frizzled receptor	HSC	hepatic stellate cells
G-CSF	Granulocyte colony-stimulating factor	Hsp	Heat-shock protein
G <sub>0</sub> , G <sub>1</sub> , G <sub>2</sub>	Gap phases of the cell cycle	HSV	Herpes simplex virus
g	Acceleration due to gravity	HVDRR	Hereditary vitamin-D-resistant rickets
g	Gram	HVO	Hypovitaminosis D osteopathy
GABP $\alpha$	GA binding protein transcription factor $\alpha$	i.m.	Intramuscular
GAD65	Glutamic acid decarboxylase 65-kDa	i.p.	Intraperitoneal
GADD45	Growth arrest and DNA damage inducible	i.v.	Intravenous
GAG	Glycosaminoglycan	IA-2	Islet antigen 2
GC-MS	Gas chromatography–mass spectrometry	IBD	Inflammatory bowel disease
GDF15	growth/differentiation factor-15	IBMX	Isobutylmethylxanthine
GDNF	Glial-cell-derived neurotrophic factor	IC <sub>50</sub>	Concentration to inhibit 50% effect
GFP	Green fluorescent protein	ICA	Intestinal calcium absorption
GFR	Glomerular filtration rate	ICMA	Immunochemiluminometric assay
GH	Growth hormone	ICOS	Inducible T-cell costimulator
GHRH	Growth-hormone-releasing hormone	IDBP	Intracellular vitamin-D-binding protein
GIO	Glucocorticoid-induced osteoporosis	IDDM	Insulin-dependent diabetes mellitus
Gli1	Glioma-associated oncogene homolog	IDH1	Isocitrate dehydrogenase 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor	IDM	Infants of diabetic mothers
GnRH	Gonadotropin-releasing hormone	IEL	Intraepithelial cells
GR	Glucocorticoid receptor	IFN	Interferon
GRE	Glucocorticoid response element	Ig	Immunoglobulin
GRTH	Generalized resistance to thyroid hormone	IGF-IR	IGF-I receptor
GSH	Glutathione	IGF	Insulin-like growth factor
GSK	Glycogen synthase kinase 3	IGFBP	IGF-binding protein
GWAS	Genome-wide association study	IGV	Integrative Genomics Viewer
h	Hour	IKK $\beta$	I kappa B kinase beta
HAT	Histone acetyltransferase	IL	Interleukin (e.g., IL-1, IL-1 $\beta$ , etc.)
HB	Hepatitis B	IMCaI	Intestinal membrane calcium-binding complex
hCAP-18	human cationic antimicrobial protein	iNKT	Invariant NKT
HCC	hepatocellular carcinoma	iNOS	Inducible nitric oxide synthase
HCV	chronic hepatitis C virus	IP <sub>3</sub>	Inositol trisphosphate
HCY	Homocysteine	IRMA	Immunoradiometric assay
HDAC	Histone deacetylase	IU	International units
HEK	Human embryonic kidney		

IUPAC International Union of Pure and Applied Chemists	MET Methionine
I $\kappa$ B Inhibitor of nuclear factor kappa B	MGP Matrix Gla protein
JAK Janus kinase	MHC Major histocompatibility complex
JG Juxtaglomerular	min Minute
JNK c-Jun NH <sub>2</sub> -terminal kinase	MIU Million international units
JNK c-Jun N-terminal kinase	MK/TEI (23S)-25-dehydro-1 $\alpha$ -OH-D3-26,23-lactone
kb Kilobases	MKP5 Mitogen-activated protein kinase phosphatase 5
kbp Kilobase pairs	MLR Mixed lymphocyte reaction
Kd Dissociation constant	Mlt Mineralization lag time
kDa Kilodaltons	MMF Mycophenolate mofetil
KDM Lysine demethylase	MMP Matrix metalloproteinase
Keap1 Kelch-like ECH-associated protein	MR Mineralocorticoid receptor
K <sub>m</sub> Michaelis constant	MRI Magnetic resonance imaging
KMT Lysine methyltransferase	mRNA Messenger ribonucleic acid
KO Knockout	MRP4 Multidrug resistance-associated protein 4
KRA Kirsten ras oncogene homolog	MS Multiple sclerosis
KRAS V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	MT Metric ton
LADA Latent autoimmune diabetes in adults	MTC Medullary thyroid carcinoma
LBD Ligand-binding domain	MTHFR N,N-methylene-tetrahydrofolate reductase
LC-MS/MS Liquid chromatography tandem mass spectrometry	mTOR Mammalian target of rapamycin
LCA Lithocholic acid	NADH Nicotinamide adenine dinucleotide
LDL Low-density lipoprotein	NADPH Nicotinamide adenine dinucleotide phosphate
LDLRAP1 Low-density lipoprotein receptor adaptor protein 1	NAF Nuclear accessory factor
Lef Lymphoid enhancer factor	NAFLD Nonalcoholic fatty liver disease
LFNG LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyl transferase	NASH Nonalcoholic steatohepatitis
Li. Ce Lining cell	NBT Nitroblue tetrazolium
LIF Leukemia inhibitory factor	NcAMP Nephrogenous cAMP
LNH Late neonatal hypocalcemia	NCCN National Comprehensive Cancer Network
LOD Logarithm of the odds	NCP Noncollagen protein
LPR6 Low-density lipoprotein receptor 6	NER Nucleotide excision repair
LPS Lipopolysaccharide	NF $\kappa$ B Nuclear factor kappa B
LSCC Lung squamous cell carcinoma	NGF Nerve growth factor
LT Leukotriene	NHANES III National Health and Nutrition Examination Survey III
LUAD Lung adenocarcinoma	NHL Non-Hodgkin's lymphoma
LXR Liver X receptor	NIDDM Noninsulin-dependent diabetes mellitus
M-CSF Macrophage colony-stimulating factor	NIH National Institutes of Health
M Mitosis phase of cell cycle	NK cell Natural killer cell
M Molar	NLS Nuclear localization signal
Mab Monoclonal antibody	NMR Nuclear magnetic resonance
MAPK Mitogen-activated protein kinase	NOD Nod-like
MAR Matrix attachment region	NOD Nonobese diabetic
MAR Mineral apposition rate	NPT Sodium/phosphate cotransporter
MARRS Membrane-associated rapid response steroid	NR Nuclear receptor
MBP Myelin basic protein	Nrf2 Nuclear factor-erythroid-2-related factor 2
MCR Metabolic clearance rate	NSCLC Non-small-cell lung cancer
MEK Mitogen-activated ERK kinase	Ob Osteoblast
MEN2 Multiple endocrine neoplasia type 2	Oc Osteocalcin or osteoclast
	OCIF Osteoclastogenesis inhibitory factor (same as OPG)
	OCT 22-Oxacalcitriol
	ODF Osteoclast differentiation factor (same as RANKL)

OHO	Oncogenic hypophosphatemic osteomalacia	PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
OLG	Oligodendrocyte	PLC	Phospholipase C
Omt	Osteoid maturation time	PMA	Phorbol 12-myristate 13-acetate
OPC	Oligodendrocyte precursor cell	PMCA	Plasma membrane calcium pump
OPG	Osteoprotegerin	PMH	Past medical history
OPN	Osteopontin	poly(A)	Polyadenosine
OSM	Oncostatin M	PPAR	Peroxisome proliferator-activated receptor
OVX	Ovariectomy	PR	Progesterone receptor
p.o.	Oral	PRA	Plasma renin activity
p21	Cyclin-dependent kinase inhibitor 1A	PRL	Prolactin
p27	Cyclin-dependent kinase inhibitor 1B	PRR	Pattern recognition receptors
p53	Transformation-related protein 53	PSA	Prostate-specific antigen
PA <sub>2</sub>	Phospholipase A <sub>2</sub>	PSC	Pancreatic stellate cells
PAD	Peripheral arterial vascular disease	PSI	Psoriasis severity index
PAM	Pulmonary alveolar macrophage	PT	Parathyroid
PAMP	Pathogen associated molecular pattern	Ptch	Patched
PARP	Poly(ADP-ribose) polymerase (PARP)	PTEN	Phosphatase and tensin homolog
PBAF	Polybromo and BRG-1 associated factors	PTH	Parathyroid hormone
PBL	Peripheral blood lymphocyte	PTHrP	Parathyroid hormone-related peptide
PBMC	Peripheral blood mononuclear cells	PTX	Parathyroidectomy
PBS	Phosphate-buffered saline	PUVA	Psoralen-ultraviolet A
PC	Phosphatidylcholine	QCT	Quantitative computerized tomography
PCa	Prostate cancer	QSAR	Quantitative structure-activity relationship
PCNA	Proliferating cell nuclear antigen	QW 1	-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitamin D3 = QW-1624F2-2
PCR	Polymerase chain reaction	RA	Retinoic acid
PCT	Proximal convoluted tubule	RA	Rheumatoid arthritis
PD-L1	Programmed death-ligand 1	Rac	Ras-related C3 botulinum toxin substrate
PDAC	pancreatic ductal adenocarcinoma	Rag	Recombination-activating gene
PDDR	Pseudovitamin D deficiency rickets	RANK	Receptor activator NF-κB
PDGF	Platelet-derived growth factor	RANKL	Receptor activator NF-κB ligand
PDGFR	Platelet-derived growth factor receptor	RAP	Receptor-associated protein
PEIT	Percutaneous ethanol injection therapy	RAR	Retinoic acid receptor
PERK	Protein kinase RNA-like endoplasmic reticulum kinase	RARE	Retinoic acid response element
PFS	Progression free survival	Ras	Rat sarcoma virus
PG	Prostaglandin	RAS	Rennin-angiotensin system
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>	RBP	Retinol-binding protein
PGF <sub>2α</sub>	Prostaglandin F <sub>2α</sub>	RCI	Relative competitive index
PHA	Phytohemagglutinin	RDA	Recommended dietary allowance
PHEX	Phosphate-regulating gene with homologies to endopeptidases on the X chromosome	RFLP	Restriction fragment length polymorphism
PHP	Pseudohypoparathyroidism	RFS	Recurrence free survival
PI3K	Phosphatidylinositol 3-kinase	RHO	Rhodopsin
P <sub>i</sub>	Inorganic phosphate	RIA	Radioimmunoassay
PIA	Proliferative inflammatory atrophy	RID	Receptor interacting domain
PIC	Preinitiation complex	RNA-seq	RNA sequencing
PIN	Prostate intraepithelial neoplasia	RNase	Ribonuclease
PIP2	Phosphatidylinositol-4,5-bisphosphate (PIP2)	RNS	Reactive nitrogen species
PIP3	Phosphatidylinositol-3,4,5-trisphosphate (PIP3)	ROCK	Rho-associated protein kinase
PKA	Protein kinase A	ROCs	Receptor-operated calcium channels
PKC	Protein kinase C	ROS	Reactive oxygen species
PKCζ	Protein kinase C zeta	RPA	Replication protein A
PKI	Protein kinase inhibitor	RPA	Ribonuclease protection assay
		RRA	Radioreceptor assay



<b>RRMS</b> Relapsing-remitting multiple sclerosis	<b>TGF</b> Transforming growth factor
<b>RT-PCR</b> Reverse transcriptase-polymerase chain reaction	<b>Th17</b> T helper type-17
<b>RUNX2</b> RUNX family transcription factor 2	<b>Th1</b> T helper type-1
<b>RXR</b> Retinoid X receptor	<b>Th</b> T helper cells
<b>RXRE</b> Retinoid X receptor response element	<b>TIO</b> Tumor-induced osteomalacia
<b>SARS-2/COVID-19</b> severe acute respiratory syndrome/coronavirus disease	<b>TK</b> Thymidine kinase
<b>SBP</b> Systolic blood pressure	<b>TLR</b> Toll-like receptor
<b>SCC</b> Squamous cell carcinoma	<b>TmP or TmPi</b> Tubular absorptive maximum for phosphorus
<b>SLC</b> Small cell lung cancer	<b>TNBS</b> Trinitrobenzene sulfonic acid
<b>SCUP-h</b> Skin Cancer Utrecht-Philadelphia-human (SCUP-h)	<b>TNFR</b> Tumor necrosis factor receptor
<b>SD</b> Standard deviation	<b>TNF<math>\alpha</math></b> Tumor necrosis factor alpha
<b>SDS</b> Sodium dodecyl sulfate	<b>TP53</b> Tumor protein P53
<b>SE</b> Standard error	<b>TPA</b> 12-O-tetradecanoylphorbol-13-acetate
<b>SEM</b> Standard error of the mean	<b>TPN</b> Total parenteral nutrition
<b>SH</b> Social history	<b>TPTX</b> Thyroparathyroidectomized
<b>SHBG</b> Sex-hormone-binding globulin	<b>T<math>\beta</math>1</b> T regulatory type-1
<b>SLE</b> Systemic lupus erythematosus	<b>TR</b> Thyroid hormone receptor
<b>SMAD4</b> Mothers against decapentaplegic homolog 4	<b>TRAP</b> Tartrate-resistant acid phosphatase
<b>Smo</b> Smoothened	<b>TRAP</b> Thyroid hormone receptor-associated proteins
<b>SNAIL</b> SNAIL Drosophila homolog of 1	<b>TRE</b> Thyroid hormone response element
<b>SOS</b> Speed of sound	<b>TRE</b> TPA response element
<b>SOX9</b> SRY(sex-determining region Y)-box 9	<b>Treg</b> Regulatory T cell
<b>Sp1</b> Selective promoter factor 1	<b>TRH</b> Thyrotropin-releasing hormone
<b>SPF</b> Sun protection factor	<b>TRIGR</b> Trial to reduce IDDM in the genetically at risk
<b>SPI1</b> Spleen focus forming virus (SFFV) proviral integration oncogene, also called PU.1	<b>Trk</b> Tyrosine kinase
<b>SRC-1</b> Steroid receptor coactivator-1	<b>TRP</b> Transient receptor potential
<b>SSCP</b> Single-strand conformational polymorphism	<b>TRPC6</b> Transient receptor potential cation channel subfamily C member 6
<b>STAT3</b> Signal transducer and activator of transcription 3	<b>TSDR</b> Treg-specific demethylated region
<b>STZ</b> Streptozotocin	<b>TSH</b> Thyrotropin
<b>SV40</b> Simian virus 40	<b>TSS</b> Transcription start site
<b>SXA</b> Single energy X-ray absorptiometry	<b>TWIST</b> Twist family basic helix-loop-helix transcription factor
<b>t<sub>1/2</sub></b> Half-life	<b>UC</b> Ulcerative colitis
<b>T1D</b> Type 1 diabetes	<b>UF</b> Ultrafiltrable fluid
<b>T<sub>3</sub></b> Triiodothyronine	<b>US</b> Ultrasonography
<b>T<sub>4</sub></b> Thyroxine	<b>USDA</b> US Department of Agriculture
<b>T4N5</b> T4 endonuclease 5	<b>UTR</b> Untranslated region
<b>TAD</b> Topologically associating domain	<b>UV-DDB</b> UV-damaged DNA-binding protein
<b>TAF</b> Tumor-associated fibroblasts	<b>UV</b> Ultraviolet radiation
<b>TBG</b> Thyroid-binding globulin	<b>UVA</b> Ultraviolet A radiation
<b>TBP</b> TATA-binding protein	<b>UVB</b> Ultraviolet B radiation
<b>TC</b> Tumoral calcinosis	<b>VDDR-I</b> Vitamin-D-dependent rickets type I ( <i>see</i> PDDR)
<b>Tcf</b> T cell factor	<b>VDDR-II</b> Vitamin-D-dependent rickets type II ( <i>see</i> HVDRR)
<b>TEDDY</b> The Environmental Determinants of Diabetes in the Young	<b>VDR</b> Vitamin D receptor
<b>TET</b> Ten-eleven translocation	<b>VDRE</b> Vitamin D response element
<b>TF</b> Tubular fluid	<b>VDRL</b> Vitamin D receptor ligand
<b>TFIIB</b> General transcription factor IIB	<b>VEGF</b> Vascular endothelial growth factor
<b>TG</b> Transgenic	<b>VERT</b> Vertebral Efficacy with Risedronate Therapy studies

VICCs	Voltage-insensitive calcium channels	WT	Wild-type
ViDA	Vitamin D assessment randomized clinical trial	XLH	X-linked hypophosphatemic rickets
VIM	Vimentin	XP	Xeroderma pigmentosum
VITAL	Vitamin D and Omega-3 Trial	XPC	Xeroderma pigmentosum complementation group
VSMC	Vascular smooth muscle cell	C	protein
VSSCs	Voltage-sensitive calcium channels	XRD	X-ray diffraction
WHI	Women's Health Initiative	YAP-1	Yes-associated protein 1
WNT	Wingless-related integration site	ZEB	Zinc finger, E box-binding transcription factor
WRE	Wilms' tumor gene, WT1, responsive element	ZnT8	Zinc transporter 8
WSTF	Williams syndrome transcription factor		



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# Relevant lab values in adults and children

## CRITERIA FOR VITAMIN D DEFICIENCY: 25(OH)D SERUM LEVELS

### Recommendations for adults

Institute of Medicine recommendations

	Conventional units (ng/mL)	SI units (nmol/L)
Deficient	<20	<50
Normal	≥20	≥50
Excessive	>50	>125

Frequently used vitamin D cut points by many laboratories similar to the Endocrine Society guidelines

	Conventional units (ng/mL)	SI units (nmol/L)
Deficient	<20	<50
Insufficient	20–29.9	50–74.9
Sufficient	30	>75

### Recommendations for pediatrics

nmol/L	ng/mL	Journal of Clinical Endocrinology and Metabolism <sup>a</sup>	Nature Rev Endo <sup>b</sup>
>50	20	Sufficiency	Sufficiency
30–50	12–20	Insufficiency	Deficiency
<30	12	Deficiency	Severe deficiency

<sup>a</sup>Munns CF, et al. Global consensus recommendations on prevention and management of nutritional rickets. J Clin Endocrinol Metab 2016;101:394–415.

<sup>b</sup>Bouillon R. Nat Rev Endocrinol August 2017;13(8):466–479.

Approximate normal ranges for serum values in adults<sup>a</sup>

Measure	Conventional units	SI units	Conversion factor <sup>b</sup>
Ionized calcium	4.5–53 mg/dL	1.12–1.32 mmol/L	0.2495
Total calcium	8.7–10.1 mg/dL	2.17–2.52 mmol/L	0.2495
Phosphorous, inorganic	2.4–4.6 mg/dL	0.77–1.49 mol/L	0.3229
1,25(OH) <sub>2</sub> D	25–45 pg/mL	60–108 pmol/L	2.40

<sup>a</sup>Normal ranges differ in various laboratories and these values are provided only as a general guide.

<sup>b</sup>Conversion factor × conventional units = SI units.

Approximate normal ranges for serum values in children<sup>a</sup>

Measure	Conventional units	SI units	Conversion factor <sup>b</sup>
Ionized calcium	4.8–52 mg/dL	1.19–1.29 mmol/L	0.2495
Total calcium	9.0–10.5 mg/dL	2.25–2.63 mmol/L	0.2495
Phosphorous, inorganic	3.8–5.0 mg/dL	1.23–1.62 mol/L	0.3229
1,25(OH) <sub>2</sub> D	27–56 pg/mL	65–134 pmol/L	2.40

<sup>a</sup>Normal ranges differ in various laboratories and these values are provided only as a general guide.

<sup>b</sup>Conversion factor × conventional units = SI units.

### Useful equivalencies of different units

Vitamin D	1 µg = 40 IU
Calcium	1 mmol = 40 mg
Phosphorus	1 mmol = 30 mg

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# Defining thresholds for vitamin D I: scientific rationale for serum 25-hydroxyvitamin D cutoffs of 25 and 50 nmol/L

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## OBJECTIVES

- To discuss the case for the use of the 25 nmol/L cutoff, with particular relevance for ensuring musculoskeletal health.
- To discuss the case for the use of the 50 nmol/L cutoff, with particular relevance for ensuring cellular health, including adequate immune function and prevention of cancer.
- To discuss the needs of specific population groups (e.g., those from ethnic minority groups, older people, those with certain health conditions, those taking certain medications, and those pregnant or lactating) who may have difficulties in meeting desirable 25(OH)D concentration due to lifestyle, health, or physiological factors, or who may even require a different 25(OH)D threshold.
- To discuss the issues surrounding meaningful definition of thresholds for vitamin D.

## 1. Introduction

There has been intense debate regarding the required level of serum or plasma 25-hydroxyvitamin D (25(OH)D), which must be achieved, in humans, to avoid vitamin D deficiency and insufficiency, as well as to provide optimal health for all tissue types. In this chapter,

the evidence for setting the thresholds of 25 and 50 nmol/L will be put forward, including the current recommendations by institutions such as the US Institute of Medicine (IOM) [1], the European Food Standards Agency (EFSA) [2], and the UK Scientific Advisory Committee on Nutrition (SACN) [3]. The chapter will also discuss special considerations for some population groups in meeting 25(OH)D thresholds, including ethnic minorities, older individuals, those who are pregnant or lactating, and those with certain health conditions or medications. Issues affecting the ability to derive meaningful thresholds will also be discussed.

## 2. Justification for serum 25(OH)D concentration >25 nmol/L

### 2.1 The UK scientific advisory committee on nutrition

In 2016, SACN recommended that the UK population should have a 25(OH)D concentration above 25 nmol/L throughout the year [3]. They highlighted that some population groups may be at particular risk of having 25(OH)D < 25 nmol/L due to low summer sun exposure, having darker skin, or wearing clothes that cover the skin year-round [3]. Recommending a sun exposure for the whole population was deemed not possible due to the complexity of this requirement considering the wide range of factors affecting vitamin D status [3].

In terms of vitamin D intake, SACN proposed a reference nutrient intake (RNI) of 10 µg per day, year-round, for those aged 4 years and over, which would ensure 25(OH)D concentrations over 25 nmol/L in 97.5% of the population, when sun exposure is minimal [3]. Safe intakes were recommended for those under 4 years, as there was not enough data in these age groups to calculate RNIs [3]. Specifically, these were as follows: 8.5–10 µg per day (age 0–1 year, as well as breastfed [partially or exclusively] infants from birth); 10 µg per day (ages 1–4 years) [3]. It was recognized by SACN (2016) [3] that 10 µg is difficult to achieve from diet alone and that other strategies should be considered to enable the population to meet these required intakes [3].

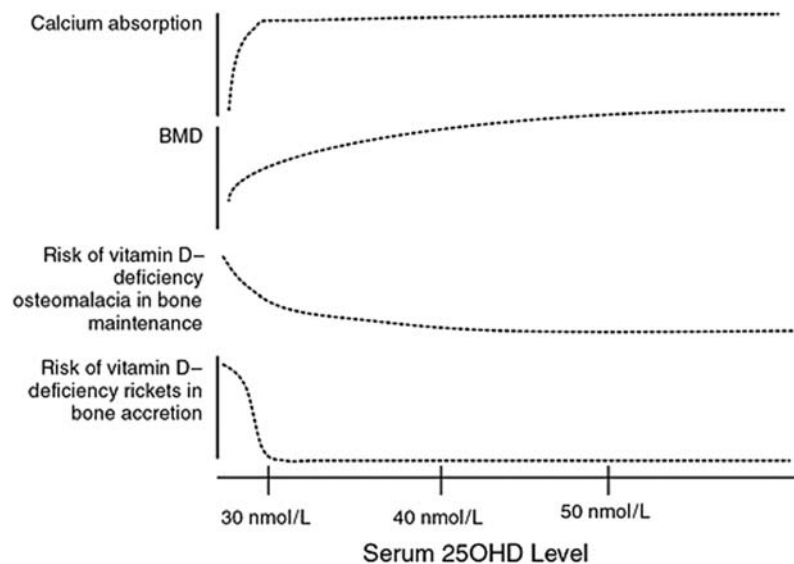
The recommendation for >25 nmol/L (and the concordant RNIs and safe intakes required to achieve this) was based on the requirements for 25(OH)D to ensure adequate musculoskeletal health in both adults and children [3]. SACN deemed that there was not enough evidence, including that from randomized control trials, regarding nonmusculoskeletal health outcomes. Of note, this differed from the approaches taken by the EFSA [2] and the Nordic Guidelines [4], whereby at least one nonmusculoskeletal outcome was used to ascertain required 25(OH)D cutoffs.

In terms of nonmusculoskeletal health outcomes, evidence was systematically reviewed, but not used in the decision-making regarding the final threshold chosen. These health outcomes included reproductive health (maternal and child outcomes), cardiovascular disease, hypertension, cancer, immune modulation, neuropsychological functioning, infectious diseases, oral health, all-cause mortality, and age-related macular degeneration. For pregnancy, links between gestational diabetes with 25(OH)D were unclear, but some randomized control trials suggested vitamin D, in combination with calcium, may reduce the risk of preeclampsia [3]. In terms of cancer, with the exception of colorectal sites, there was no strong association between 25(OH)D and cancer risk. Moreover, few randomized controlled trials conducted showed no effect of vitamin D supplementation on cancer risk [3]. For CVD, although cohort studies showed a reduced risk of CVD with increased 25(OH)D, intervention trials showed no association [3]. For hypertension, cohort and cross-sectional studies showed a negative association between 25(OH)D and hypertension, but randomized control trial metaanalyses showed inconsistent results [3]. For all-cause mortality, observational studies suggested a negative association between mortality and serum 25(OH)D, but a systematic review of randomized control trials showed no effect of vitamin D intake or vitamin D status on mortality [3]. In terms of

immunity, cohort studies on asthma and other atopic disorders gave inconsistent findings, and there was little evidence that vitamin D supplementation had any effect on risk of developing autoimmune disease [3]. There was little evidence assessing the risk of developing type 1 diabetes, rheumatoid arthritis, inflammatory bowel disease, and systemic lupus erythematosus (SLE) [3], and evidence for the link between serum 25(OH)D and multiple sclerosis was inconsistent [3]. For respiratory tract infections (RTIs), cohort studies suggested an inverse association with 25(OH)D risk, with thresholds for increased risk at either <25 nmol/L or <50 nmol/L [3]. There was little evidence to support a link between 25(OH)D and mental health with some studies being of ecological design [3]. There was some evidence that oral health is linked to 25(OH)D, potentially via effects on tooth structure, but for periodontal disease, it was suggested that it may partly be via associations between 25(OH)D and immune health [3]. Finally, there were no studies on the association between 25(OH)D and age-related macular degeneration [3].

For musculoskeletal health, SACN was not able to discern, based on current evidence, a threshold below which rickets occurs ([3]). However, in most case reports and other observational studies, 25(OH)D was <25 nmol/L, and this was used to support the cut-off of 25 nmol/L for musculoskeletal health, for the purposes of population health [3]. It was acknowledged that this is not a specific clinical diagnostic threshold [3]. In terms of osteomalacia, most data were from case reports and two cross-sectional studies, with 25(OH)D ranging from 4 to 20 nmol/L [3], again supporting a threshold of 25 nmol/L for musculoskeletal health.

Conversely, some evidence suggested that a 25(OH)D higher than 25 nmol/L is required for skeletal health [5], with a higher requirement of at least 50 nmol/L predicted to ensure that 97.5% of the population is protected against osteomalacia [5], and potentially even 75 nmol/L required to fully mineralize bone tissue [5]. However, this was based on the amount of osteoid in bone from biopsies taken during postmortem examinations and correlated with past 25(OH)D status measurements from medical records. Therefore, the 25(OH)D result was not necessarily accurate for the time of death. Critique of the limitations of using postmortem bone data has been given in more detail by Aspray and Francis (2013) [6], and the results from the postmortem study were not considered reliable enough data by SACN to steer the decision toward 50 nmol/L as the cutoff for musculoskeletal health. This is in contrast to the decision-made by the US IOM [1], who considered the postmortem data [5] sufficient evidence for supporting the 50 nmol/L cutoff for musculoskeletal health.



**FIGURE 51.1** IOM: Conceptualization of integrated bone health outcomes and vitamin D exposure. Bone; calcium; 25(OH)D, 25-hydroxyvitamin D. Source: IOM report [1], page 368. Reproduced with permission from the National Academies Press.

### 3. Justification for serum 25(OH)D concentration >50 nmol/L

#### 3.1 The US/Canada Institute of Medicine

A 25(OH)D concentration greater than 50 nmol/L for population health has been recommended by the US IOM [1], and EFSA [2], as well as the Nordic guidelines [4]. The cutoff of 50 nmol/L was based on three main threads of evidence for ensuring bone health, assuming adequate calcium intake (Fig. 51.1). First, risk of rickets is minimized when 25(OH)D status is between 30 and 50 nmol/L [1]. Second, randomized trials and observational studies assessing adult fracture risk suggest a 25(OH)D status of 40–50 nmol/L is required to reduce fracture risk, albeit some studies noted that 60–70 nmol/L was superior [1]. Third, the postmortem data, as discussed before, support a threshold of at least 50 nmol/L [1,5]. The focus on skeletal health was due to the lack of evidence of other health outcomes, as judged by the IOM Committee.

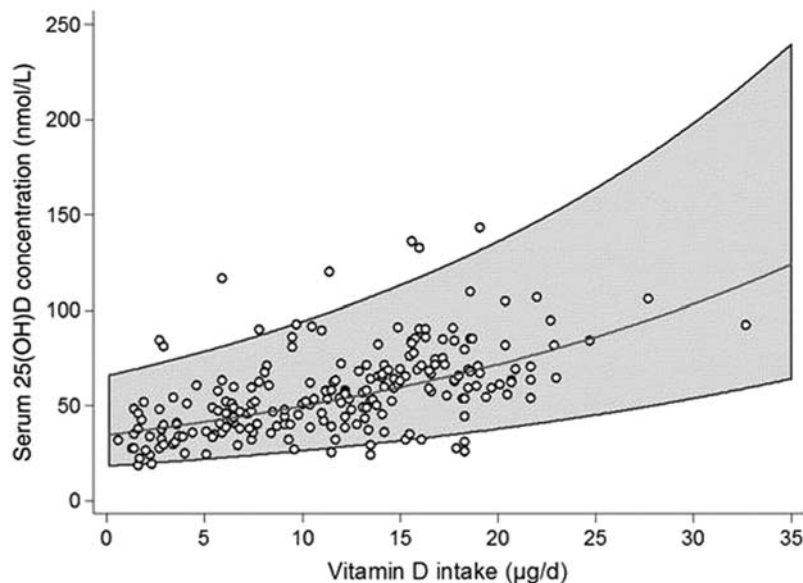
The IOM used the principle that 25(OH)D concentration is correlated to vitamin D intake, so intake data are useful in simulating dose–response curves when sun exposure is low [1]. This was then used to estimate the intake required to maintain population 25(OH)D at the level that modeling suggested was required for bone health (50 nmol/L) [1] (Fig. 51.2).

The IOM based their dietary reference intakes (DRIs) on achieving 50 nmol/L to cover the needs of 97.5% of

the population, and set Recommended Dietary Allowances (RDAs) for those aged over 1 year and adequate intakes (AIs) for those aged 1 year or younger [1]. The IOM noted a lack of dose–response intervention trials in the literature base, with most trials being only single dose, and often not reporting background dietary intake of vitamin D [1]. In addition, many trials supplemented both vitamin D and calcium, so it was difficult to assess the separate effects of each nutrient separately on the health outcome being assessed [1]. The DRIs for vitamin D were based on those required when calcium was adequate and sun exposure was minimal [1]. The criteria of minimal sun exposure were important as UVB exposure is such a complex construct, and there was reluctance to assume a certain degree of sun exposure when setting dietary requirements [1]. The AI for infants aged up to 12 months was set as 400IU/d, with an RDA of 600IU/d for those aged 1–70 years, and an RDA of 800IU/d for the over seventies. For pregnancy and lactation, 600IU/d was set as the RDA.

The IOM report presented tolerable upper intake levels (UL) for different life stages as follows: 1000 IU (25 µg) per day for age 0–6 months, 1500 IU (38 µg) per day for age 6–12 months, 2500 IU (63 µg) per day for age 1–3 years, 3000 IU (75 µg per day) for age 4–8 years, and 4000 IU (100 µg) per day for 9 years and older [1].

A plethora of research articles supporting the 50 nmol/L cutoff have been published since the release of the IOM report, including in relation to



**FIGURE 51.2** The relation between serum 25-hydroxyvitamin D [25(OH)D] concentrations (in late winter 2007) and total vitamin D intake (diet and supplemental) in 20- to 40-year-old healthy persons ( $n = 215$ ) living at northerly latitudes ( $51$  and  $55^{\circ}\text{N}$ ). Mean response and 95% CIs in the shaded area. Vitamin D intake; dietary vitamin D; 25(OH)D, 25-hydroxyvitamin D. Source: Cashman et al. [7]. Copyright (2008), with permission from Elsevier.

nonmusculoskeletal outcomes. Indeed, it is possible that a higher 25(OH)D than 25 nmol/L is needed for health of other tissue types, including immune cells and general cell health to prevent cancer. For example, numerous studies have found a benefit for reduction of immune-associated disease when 25(OH)D  $> 50$  nmol/L, after controlling for key confounders, including pneumonia survival [8] and risk of developing acute respiratory tract infections [9–11]. In terms of cancer, studies have found 25(OH)D greater than 50 nmol/L was associated with higher breast cancer survival [12] and lower risk of colorectal cancer [13], albeit some studies have found that 75 nmol/L is more beneficial for reducing cancer risk, for example, the Longitudinal Aging Study Amsterdam [14]. For pregnancy-related outcomes, having maternal 25(OH)D  $> 50$  nmol/L has been associated with reduced risk of preeclampsia [15,16], and higher risk of adverse lung and neurocognitive development [17] as well as poorer bone outcomes [18] in the offspring.

For mortality, a recent paper using UK Biobank data in 365,530 participants, with no CVD, diabetes, or cancer history at baseline (2006–10), found reduced cancer-specific mortality with baseline 25(OH)D  $> 45$  nmol/L as well as reduced all-cause and CVD mortality with baseline 25(OH)D  $> 60$  nmol/L [19]. In addition, multiple studies show that the PTH suppression (plateau), associated with 25(OH)D, occurs at around 30–50 nmol/L [20,21]. Most recently, nonlinear Mendelian randomization studies have suggested vitamin D

status is causally linked to risk of CVD and all-cause mortality, with a leveling off of risk for both of these outcomes at around 50 nmol/L [22,23].

However, the methods used in the IOM report [1] have been criticized. The review by Heaney and Holick [24] highlights evidence favoring a cutoff of 75 nmol/L for bone health, citing evidence from randomized control trials, including metaanalyses of randomized control trials, which showed further fracture reduction after vitamin D supplementation up to 75 nmol/L [24]. Moreover, the review suggests a different interpretation of the Priemel et al. data [5], in which 75 nmol/L more closely matches the point at which bone osteoid is minimized. The review also points out that 600 IU does not align with the known rule of thumb for how vitamin D intake affects 25(OH)D (each extra 100 IU/d puts 25(OH)D up by 2.5 nmol/L) and the DRIs do not account for the fact the people with obesity need larger amounts of vitamin D than those without obesity [24].

As pointed out in another commentary [25], the IOM decision differs from recommendations given by international bodies such as the International Osteoporosis Foundation (IOF), who recommend 800–1000 IU/d for those aged 60 years and over, and see 75 nmol/L as the recommended 25(OH)D concentration to prevent falls and fractures [26]. This commentary [25] discusses how the IOF recommends 700–1000 IU/d for falls prevention [26], but the 50 nmol/L cutoff was still derived solely from bone health outcomes.



### 3.2 European food safety agency and nordic guidelines

When setting their recommendation in 2016, EFSA assumed minimal sun exposure and used serum 25(OH)D as their biomarker of vitamin D status [2]. The health outcomes they used to set the DRV included musculoskeletal and pregnancy-related outcomes, but not lactation-related health outcomes (due to a lack of a relationship with 25(OH)D) or other nonmusculoskeletal outcomes (due to insufficient evidence) [2]. The data suggested increased risk of adverse effects for both musculoskeletal health and pregnancy-related health below 50 nmol/L [2]. Using data from randomized control trials, they set an adequate intake (AI) of 15 µg per day for those aged 1 year and older and 10 µg per day for infants aged 7–11 months. The AI for pregnancy and lactation was 15 µg per day [2].

The Nordic Nutrition Recommendations (2012) [4] defined vitamin D insufficiency as 25(OH)D 30–50 nmol/L, and defining 50 nmol/L as the threshold for sufficiency. To achieve this in the population, they set a recommendation for 10 µg per day for ages for first weeks of life to age 74 years (no adjustment for pregnancy or lactation), and 20 µg per day for 75 years and older (increased relative to younger adults due to lesser ability to produce vitamin D in the skin, and due to evidence supporting use of vitamin D to prevent fractures and falls, as well as general mortality) [4]. People with little or no sun exposure were recommended 20 µg per day [4].

To summarize, the IOM, EFSA, and Nordic Guidelines all suggested 50 nmol/L as the desired 25(OH)D threshold. They all based their recommendations on the basis of systematic literature reviews, but differed in the health outcomes considered. It must be noted that there is some justification for considering higher thresholds, for example, the US Endocrine Society Guidelines recommend 75 nmol/L [27]. In support of this, in a recent study of Brazilian women, PTH plateaued at a 25(OH)D of 70–80 nmol/L [28], albeit the slope was still relatively flat at 50 nmol/L [28]. However, we will not discuss the 75 nmol/L threshold in detail here as it is discussed in a separate chapter in this book.

## 4. Issues with reaching 25(OH)D thresholds for specific groups

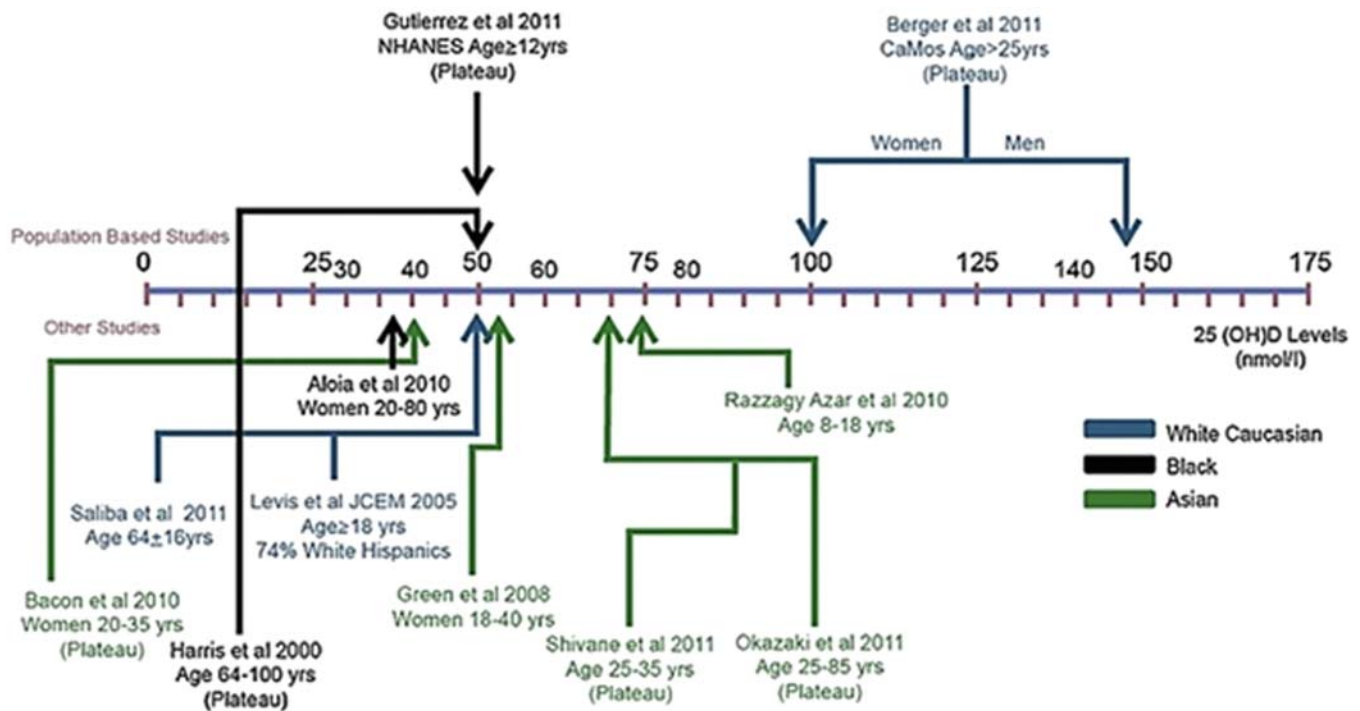
There is concern regarding the known high levels of vitamin D deficiency in darker-skinned ethnic minority groups. For example, research using the UK Biobank cohort has shown that 60% of the South Asian [29],

35% of the Black African [30], and 37% of the Black African Caribbean [30] groups (all data from 2006–10) had 25(OH)D less than 25 nmol/L. In the South Asian group of the UK Biobank, 23% of men and 39% of women took a vitamin D containing supplement (i.e., a single vitamin D supplement, and/or a multivitamin) [31]. Similar data have been found for 25(OH)D concentrations in Black and South Asian populations, living in Europe, the United States, Canada, and New Zealand [32–35]. Of concern, a recent study found that short, regular exposures to sunlight around midday in the Northern UK (53.5°N) were not enough to enable production of sufficient vitamin D in the skin of South Asian individuals [36]. Therefore, public health policies in high-latitude countries recommending short exposures to sunlight around midday, for vitamin D populations, may be insufficient for darker skinned groups.

Few data are available examining vitamin D requirements for ethnic minority groups, but a recent systematic review and metaanalysis of randomized control trials estimated that a daily intake of 67 µg per day would be required to keep Black and South Asian populations, living in high-latitude countries, above 50 nmol/L [37]. The authors discussed how this is above the current vitamin D intake recommendations by multiple international bodies and will mean that 2.5% of Black and South Asian persons at this intake would be > 158 nmol/L [37], a level that in supplementation trials has been associated with adverse effects [37]. This suggests that for some ethnic minority groups, a higher intake of vitamin D may be required to meet the 50 nmol/L threshold, albeit more research is required on this topic.

In addition, there is some suggestion that optimal thresholds for 25(OH)D may vary by ethnic group. Fig. 51.3 illustrates the inflection point (the 25(OH)D concentration at which PTH concentration stops rising and reaches a plateau), for Asian, Black, and White populations. There is some indication that PTH plateaus at a lower level in Asian and Black populations (35–70 nmol/L) than in White populations (50–150 nmol/L) albeit the ethnic difference shown are highly dependent on the CaMos study [38], with other studies showing a more similar plateau between ethnic groups.

The ability to produce 25(OH)D in the skin declines with aging, likely due to reduced production of previtamin D under the action of ultraviolet radiation [39] as well as a declined ability to hydroxylate 25(OH)D [40,41] (albeit it is unclear as to whether the latter is due to age per se, or due to age-associated comorbidities [40]). Therefore, older people may have higher requirements for vitamin D intake, or sunlight exposure, than



**FIGURE 51.3** Inflection points for PTH in relation to 25(OH)D by ethnicity. From El-Hajj Fuleihan, Rahme and Bassil in Burckhardt, Dawson-Hughes, and Weaver (Eds). Copyright (2013), with permission from Springer Nature.

do younger people. In addition, many older people, especially those who are frail and/or in nursing homes, have little outdoor sun exposure. For example, an Australian study found nursing home residents were at 1.8 times increased risk of 25(OH)D deficiency ( $<25$  nmol/L) compared with free-living individuals [42]. Forty-seven percent of the nursing home residents had low sun exposure ( $<35$  mJ/cm<sup>2</sup>) compared with none of the free-living individuals [42].

As with younger populations, older people are likely to consume little vitamin D from the diet. The UK National Diet and Nutrition Survey (2016–19) showed a median intake of 2.4 µg vitamin D per day from food in 19- to 64-year-olds, compared with 2.9 µg per day in persons aged 65 years and older [43]. The difference is likely to be larger when considering just frail older people living in nursing homes. To summarize, older people may have lifestyle factors meaning they may have less opportunity to produce vitamin D from the sun, as well as consume it in the diet.

There is a need to be cautious regarding high 25(OH)D concentrations in persons with certain medical conditions or physiological states. Vitamin D supplementation in patients with sarcoidosis may lead to the overproduction of 1,25(OH)D<sub>2</sub> by lung macrophages, leading to hypercalcemia and hypercalciuria [44]. Persons with obesity may have altered vitamin D requirements, compared with persons with less adiposity. For example, studies have found a lower 25(OH)D in

persons with obesity [45], which may be due to sequestering of 25(OH)D by adipose tissue [46], or due to volumetric dilution due to increased body size [47]. Randomized controlled trials show that persons with obesity require a higher intake of vitamin D, compared with persons without obesity, to achieve the same 25(OH)D concentration [48]. Therefore, higher vitamin D intake requirements, to achieve a certain threshold, may be required for persons with obesity.

Finally, some medications affect vitamin D metabolism, which may affect the vitamin D requirement to meet 25(OH)D sufficiency thresholds. Use of metformin may be associated with reduced 25(OH)D status [49]. Phenytoin and sodium valproate are known to interfere with the renal 25-hydroxylation of vitamin D, which cause a problem in producing circulating 25(OH)D [50]. Thiazide diuretics reduce calcium excretion in the urine, which theoretically could alter vitamin D metabolism, although a recent review found that use of thiazides did not alter 25(OH)D concentration [51]. There has been inconsistent evidence as to whether loop or potassium-sparing diuretics have a detrimental association with vitamin D and calcium metabolism [51]. Similarly, there is inconsistent evidence as to whether statin use is associated with 25(OH)D concentration [52].

Interindividual differences (e.g., genetics) may affect vitamin D metabolism. For example, single nucleotide polymorphisms (SNPs) in vitamin D-related genes may effect actual 25(OH)D concentrations, with some

individuals potentially developing very high 25(OH)D concentrations. SNPs may also effect the optimal threshold required for each individual, via difference in cellular and tissue function. The relative prevalence of individuals who more rapidly achieve higher 25(OH)D concentrations, given the same sunlight exposure and/or dietary intakes to other people, will determine the degree of caution required by public health bodies in terms of which threshold to aim for in a population. Albeit, vitamin D genetics only contribute around 7.5% to determining 25(OH)D concentration [53], so this is likely to only be a small consideration.

## 5. Other issues surrounding definition of thresholds for vitamin D

Tissues themselves, and by extension, organs, may have differing requirements for 25(OH)D, as well as for the active hormone (1,25-dihydroxyvitamin D; 1,25(OH)<sub>2</sub>D). This is likely part of the explanation for 25 nmol/L as a minimum threshold to ensure musculoskeletal health, but 50 nmol/L to ensure health for other tissues that has been demonstrated by evidence reviewed in this chapter (e.g., immune function, anti-cancer properties).

Country-based differences in population characteristics, and statistical approach used, as well as practicalities in terms of achieving threshold (diet, supplements, fortification, sunlight) also influence the setting of thresholds. Public health bodies may differ in their willingness to proscribe what is required to meet certain thresholds, based on how feasible the threshold is in terms of required dietary intake, or sunlight exposure that is realistic.

Finally, the assessment of vitamin D status is usually by assessment of serum or plasma 25(OH)D. The active hormone (1,25(OH)<sub>2</sub>D) is less often used in research compared with 25(OH)D as 1,25(OH)<sub>2</sub>D is tightly regulated physiologically and so may be normal even when bodily stores of vitamin D may be deficient. Threshold definitions, as described before, rely heavily on the concentrations of 25(OH)D required for health. However, it is unclear to what extent serum or plasma 25(OH)D is representative of vitamin D level in the whole body (e.g., in other tissue fluids, and in the tissue itself).

## 6. Conclusion

Recommendations for 25(OH)D thresholds have been commonly set at 25 nmol/L or 50 nmol/L, with the UK SACN setting 25 nmol/L, and the US/Canadian IOM,

EFSA, and the Nordic Guidelines setting 50 nmol/L. The variation stems from differences in the criteria used to establish the threshold, factors specific to the populations linked to the guidelines, and differing interpretations of available evidence. It is important to note that some population groups are likely to have more difficulty in achieving these thresholds, including darker skinned ethnic minority groups, older people (particularly those who are frailer, or in nursing homes), and those with certain health conditions or who are on medications affected by vitamin D metabolism. These groups may need assistance in terms of reaching relevant 25(OH)D thresholds. Furthermore, there are general issues with the concept of defining 25(OH)D thresholds at all; considering 25(OH)D does not necessarily reflect tissue vitamin D content, there are issues with feasibility influencing decisions about threshold setting, and tissues may differ in their requirements for vitamin D.

## 7. Summary points

- The UK SACN currently recommends 25 nmol/L as a cut-off for 25(OH)D sufficiency, based on ensuring musculoskeletal health only.
- The US IOM, EFSA, and the Nordic Guidelines currently recommend 50 nmol/L for 25(OH)D sufficiency, for ensuring musculoskeletal health (IOM, EFSA, Nordic), pregnancy (EFSA, Nordic only)-related health, and a variety of other nonmusculoskeletal outcomes (Nordic only).
- Some population subgroups may need extra support to meet these recommended 25(OH)D thresholds.
- There are issues surrounding the definition of thresholds including varying needs for 25(OH)D and 1,25(OH)<sub>2</sub>D in different tissue types, differences between the procedures for setting guidelines between public health bodies, and issues in using serum or plasma 25(OH)D to assess actual tissue requirements for 25(OH)D.

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## Defining thresholds for vitamin D II

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### OBJECTIVES

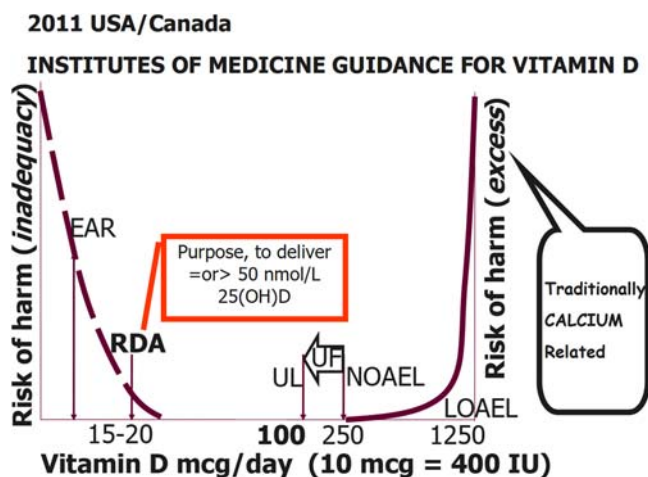
- Explain why everyone should want their serum 25(OH)D to be at least 30 ng/mL (75 nmol/L).
- Define “threshold” for vitamin D nutrition and why policy-makers misuse the concept of “evidence-based medicine.”
- Summarize the origin of current dietary guidelines for vitamin D.
- Review basic biology in the context of sunshine and vitamin D.
- Why it is impossible for nutrition clinical trials to match the quality of evidence achievable with and demanded of drug clinical trials.
- Summarize recent evidence in the context of cancer, mortality rates, multiple sclerosis risk, and life expectancy.
- The problem of COVID-19 and decisions about the role of vitamin D when there is not enough time for perfect information.
- Allay concerns about vitamin D toxicity.

### 1. Introduction

The dietary recommendation for any nutrient is based on the thinking that a “threshold” exists for the nutrient’s intake, beyond which further consumption of the nutrient delivers no additional benefit. But instead of benefit, the logic is that further consumption creates only progressively greater risk of harm [1] (Fig. 52.1).

The concept of a nutrient threshold is a trivialization that probably arose from the thinking that vitamins are simply cofactors that are needed qualitatively, so that enzymes can function properly. The concept of a threshold differs substantially from the rigors of pharmacology, in which the effects of drugs are regarded as following log–dose–response curves to produce gradually increasing effects [3]. For vitamin D, there are many putative functions related to health, and this vitamin is itself metabolized in a finely regulated manner to generate a signaling molecule, calcitriol, or 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D). The question of a threshold becomes complex: what are the requirements for the precursor, vitamin D, in the context of the multiple biological effects of its hormone, which include effects on bone and mineral health, cancer, immune function, mental health, etc. Furthermore, is the dosage threshold for vitamin D related only to the oral intake of the nutrient, or is the threshold related to a level of serum 25-hydroxyvitamin D (25(OH)D) the biochemical marker for vitamin D nutrition? To complicate these questions, what interactions are there in terms of changes in seasons of the year, sunshine, latitude, clothing, skin color, and cultural behavior?

The history of the fat-soluble vitamins extends back hundreds of years, to the time when a daily teaspoonful of cod liver oil was used in Scandinavia to help infants thrive [4,5]. The vitamin D content of that teaspoonful eventually became the basis for dietary guidance for vitamin D. A century ago, there were no dose-finding studies in clinical nutrition, and decisions were made empirically. Once it was realized that vitamin D was necessary beyond infancy to prevent osteoporosis, the use of the roughly 400 IU per day of vitamin D in a teaspoon of cod-liver oil became common throughout



**FIGURE 52.1** Summary and terminology of guidance values as they apply to vitamin D. [2], as paraphrased and interpreted by the author (RV) [1]. EAR, estimated average requirement, the intake that would satisfy the requirement for preventing the index condition (rickets or osteomalacia) for half the population, or for the average member of the population. RDA, recommended dietary allowance, the intake that would satisfy that requirement for 97.5% of the population. UL, the upper level, the long-term daily intake, beyond which the general public is advised to avoid. UF, an uncertainty factor, or margin of safety that keeps the UL well below the highest daily intake that has not been associated with harm. NOAEL, the no observed adverse event level, a daily average intake level that has not been associated with the specified measure of harm (hypercalcemia). LOAEL, the lowest observed adverse event level, an intake at which there is a clear risk of harm, in particular hypercalcemia. The specific values shown are the pertinent daily intake values for adults, as specified in the most recent IOM report [2]. Copyright R Vieth.

the lifespan, both for fortification of a quart or liter milk and for the vitamin D in supplements.

In the 1990s, clinical trials that combined tricalcium phosphate (Ostram(R)) along with vitamin D<sub>3</sub>, and that were funded by MERCK KGaA, Darmstadt, Germany, showed that 800 international units daily of vitamin D reduced rates of fractures in healthy older adults [6,7]. The sponsor was primarily interested in the calcium aspect of the research, as Pierre Meunier, the senior author of those trials, told me. The choice of the 800 IU/day of vitamin D<sub>3</sub> was based, quite simply, on his arbitrary decision to try using double the usual, 400 IU/day dose.

The 800 IU/day of vitamin D dose was chosen by Meunier, and it has now become the recommended dietary allowance for vitamin D for all people older than 70 years [2]. Other similar studies in the elderly showed that while fractures were not prevented with 400 IU/day [8], the combination of calcium with vitamin D at 700 IU/day did prevent fractures [9]. For the prevention of fractures, it does appear that 800 IU/day is the threshold beyond which clinical trials have failed to demonstrate any further benefit from giving more vitamin D [10–12]. However, if instead of an intake of

vitamin D, the focus is on a threshold for bone health and fracture prevention, then it becomes clear that the desirable minimum concentration is 30 ng/mL (75 nmol/L) [13–16].

There does remain a lack of evidence as to whether more than the 400 IU/day intake for children, which was intended to prevent rickets or osteomalacia, has longer-lasting effects at preventing fractures throughout the lifespan. It is also important to know that there no level 1 evidence that the current 600 IU/day RDA for vitamin D actually does any good at all for adults younger than the age of retirement. To conduct the lifespan-long randomized clinical trial that policy-makers want to see would be very difficult to conduct. There are clues that early-life vitamin D nutrition should benefit health in the longer term. In Lebanon, healthy adolescent girls having mean 25(OH)D of 12 ng/mL (30 nmol/L—well below the 50 nmol/L target for dietary recommendations) showed improved bone response within 1 year [17,18]. Since bone mass reaches its peak soon after age 20 years [19], and if osteoporosis is a concern, then should we not be certain to address the simple, low-hanging preventive fruit, like vitamin D, to mitigate osteoporosis later in life? During the teenage years, is it realistic to expect double-blind, placebo-controlled clinical trial evidence to test whether ensuring serum 25(OH)D levels of at least 30 ng/mL (75 nmol/L) affect peak bone mineral density? Is it wise for any parent today, to choose to wait for such evidence before adopting the target level of 30 ng/mL (75 nmol/L) for their own children? If I could make the following wager, I would be happy to do so: If a survey were taken of the people who have written chapters in this book, not even the most vocal skeptics about vitamin D would feel comfortable with the knowledge that their children or grandchildren were growing up having serum 25(OH)D levels below 75 nmol/L (30 ng/mL).

The unavoidable confounder for vitamin D nutrition research is the fact that most people acquire most of their vitamin D through exposure of their skin to ultraviolet light. That biggest problem that complicates health advice to the public is that, according to the dermatology community, “there is no such thing as a safe tan” [20]. The American Dermatology Association website states that adults should avoid sunshine and that the general public should simply adhere to the “evidence-based advice” of the Institutes of Medicine (IOM). The latest IOM report on vitamin D makes it very clear that it assumes essentially no vitamin D input from ultraviolet exposure of the skin [2]. But then, the neither the IOM nor any other agency can provide any clinical evidence for the long-term serum 25(OH)D response to a daily dose of vitamin D in people who were never exposed to sunshine. Therefore, so far as officialdom in North

America and elsewhere is concerned, the threshold for vitamin D has been reached, without level 1 evidence, by anyone adhering to official dietary guidance. Based on Bouillon's excellent comparative analysis of nutritional guidelines for vitamin D [21], the highest intakes recommended by any of the guidance authorities is as follows:

- 400 International Units (IU) for infants/children 0–1 years
- 600 IU for children, teenagers and adults 1–70 years
- 800 IU for adults 71+ years

Bear in mind, that despite official denials, the reality of those recommendations above must unavoidably be in addition to the vitamin D acquired from exposure of people to sunshine. Incidentally, the country with the world's lowest recommendation for vitamin D (100 IU/day) is the Russian Federation [21].

Is it really credible that the debate should end here? Is it correct for health policy to state that humans do not require sun exposure, and that the threshold for optimal health is ensured with an oral intake of 800 IU (20 µg)—or less—of vitamin D?

Clifford Rosen, who coauthored the chapter opposing the higher vitamin D requirement chapter in the previous edition of this volume [10,15], has been quoted in the journal, *Science*, as saying, "Evidence does not matter to many people when it comes to vitamin D," he maintains: "It is a religion. People really believe this stuff works." [22]. Others agree with Rosen's perspective that there is no evidence of net benefit from anything beyond official dietary guidelines [23–26]. A recent editorial comment by Clifford and Rosen in a high-profile journal commented on the negative fracture-prevention findings for the VITAL randomized trial of 2000 IU/day vitamin D. They concluded "Adding those findings to previous reports from VITAL and other trials showing the lack of an effect for preventing numerous conditions suggests that providers should stop screening for 25-hydroxyvitamin D levels or recommending vitamin D supplements, and people should stop taking vitamin D supplements to prevent major diseases or extend life." [125]. I regard that conclusion as disingenuous, because Clifford and Rosen must have been well aware that the healthy volunteer participants of the VITAL trial had average baseline serum 25(OH)D levels that were already at 29.3 ng/ml, which is at the 80th percentile value of the American population [82]. Those unusually high background serum 25(OH)D levels certainly satisfied any expected fracture-prevention function of vitamin D, and revealed a healthy volunteer bias for the VITAL study. I will go into more detail on this later in the cancer section of this chapter.

For formal guidelines, it seems that only the ultimate level of evidence is acceptable. But David Sackett who

led the group that developed the modern concept of "evidence-based medicine" (EBM) has clarified what he means by EBM.

"Because the randomized trial, and especially the systematic review of several randomized trials, is so much more likely to inform us and so much less likely to mislead us, it has become the "gold standard" for judging whether a treatment does more good than harm. However, some questions about therapy do not require randomized trials (successful interventions for otherwise fatal conditions) or cannot wait for the trials to be conducted. And if no randomized trial has been carried out for our patient's predicament, we must follow the trail to the next best external evidence and work from there" [27].

Clearly, evidence based medicine involves far more than just double-blind placebo-controlled clinical trials. To that, I would add that those very same official guidelines that demand the highest available evidence are in themselves subjective, and they are biased in their outcome by interpretations of evidence that depend on the very selection of the individuals chosen to review those data. By way of example, I coauthored an editorial along with 14 well-known experts in the field, calling for the IOM to review dietary guidance for vitamin D [28]. It stands as a clear example of committee-selection bias that not a single one of those 14 authors who had advocated for a review of vitamin D ended up on the panel selected for the Institute of Medicine review [2]. It is well known, that if advocates for one side of a contentious issue are excluded from decision-making, then the outcome favors the opposite side of that issue, in this case, biasing the review process in favour of the status quo.

As a glaring consequence of that bias, I direct the reader to the IOM approach to the way the committee analyzed bone biopsy data from accident fatalities in Germany, along with their serum 25(OH)D levels [29]. The IOM committee determined from its analysis of bone biopsy data of Priemel et al. that 20 ng/mL (50 nmol/L) is osteomalacia prevention threshold for serum 25(OH)D [2]. However, coverage of the debate in both the journal, *Science* [22], and the journal, *Nature* [30], shows dissenting opinions of others. Amling, the senior author of the biopsy data, disagreed strongly with the IOM analysis of the data his research group produced. The published figures of Priemel et al. show with absolute clarity that the thresholds for minimal osteomalacia, based on the observed osteoid volume, osteoid surface, and osteoid thickness are all at 30 ng/mL (75 nmol/L) [29]. Anyone who doubts the inappropriateness of the 50 nmol/L (20 ng/mL) threshold that the IOM adopted for 25(OH)D needs to take a serious look at the Priemel paper [29].

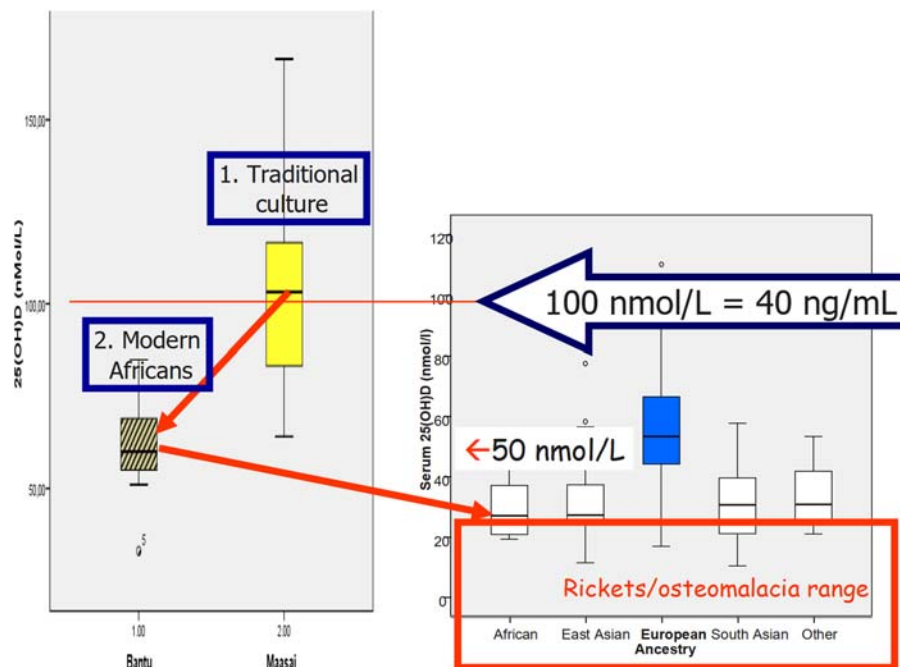
Another important point of contention relates to the methods that were used to calculate vitamin D intake

that would be needed to ensure the RDA target level for serum 25(OH)D. To allay the criticism that the committee might not be accounting for subpopulations with dark skin or older persons in institutions, the IOM guidance assumed minimal sun exposure as a basis for its vitamin D recommendations. In other words, the intent was that adherence to the RDA intake advice for vitamin D should, by itself, ensure a minimum serum 25(OH)D of 20 ng/mL (50 nmol/L) [2]. But more rigorous analyses have shown that if the aim is for 97.5% of the population to have a 25(OH)D concentration that is at least 20 ng/mL (50 nmol/L), then the most conservative of those peer-reviewed analyses concludes the intake needs to be 43.6 mcg/day (1744 IU/day) [31]. Other intake calculations to ensure 20 ng/mL (50 nmol/L) were even higher than that [31–34]. The RDA for vitamin D is still 600 IU/day for most people, an intake recommendation that does not stand up to scrutiny.

## 2. Human biology

Any evidence-based analysis of what is optimal for vitamin D should start with the basic biology of our species [35]. The fundamental concept in evolutionary biology is that it is environment during evolution that

determines genotype, and from the resulting gene pool, environmental change then drives the fine selection to affect phenotype. Evolution involves natural selection in a manner that optimizes the species' biology to suit the environment in which the species arose. Our own species originated in the horn of Africa. Early *Homo sapiens* are logically described as nudists whose skin was of type 6, on the 6-point Fitzpatrick skin type scale of skin darkness. Our species was “designed” through evolution to inhabit the tropics. The changes that accompanied the millennia-long migrations of *H. sapiens* across the various global environments resulted in selection of characteristics that maximized their ability to produce viable offspring [36–38]. While there is some debate as to the exact geographic region where the genes for whiter European skin originated [39], selection for lighter skin with distance away from the equator happened independently in both hemispheres, indicating that lighter skin color was multifactorial and driven by the environment [40,41]. Our best estimate of what serum 25(OH)D level should be regarded as biologically “normal” for *H. sapiens* is provided by Luxwolda and Muskiet who studied groups living traditionally in tropical Africa [42,43]. Fig. 52.2 shows the assembled published Luxwolda data from Africa and from healthy students at the University of



**FIGURE 52.2** Boxplots showing serum 25(OH)D nmol/L levels in of people from different ancestries in Africa (latitude 0) and during February in Toronto (43N). All results here assayed with the same Diasorin method. The Africa values are from Luxwolda and Muskiet [42,43], and these show traditionally living Masai and urban-living Bantu. Since humans originated in Africa, the Masai results logically indicate serum 25(OH)D levels that are “normal” for the human species. Levels of 25(OH)D in urban Africans match those of White Canadians, i.e., of European ancestry. People of non-European ancestry who live in north Toronto [44] exhibit half as much again, as the Masai levels. The box at the bottom right highlights 25(OH)D values at or below 25 nmol/L (10 ng/mL), which are regarded as the criterion for a diagnosis that rickets or osteomalacia was caused by vitamin D deficiency. Copyright R Vieth.



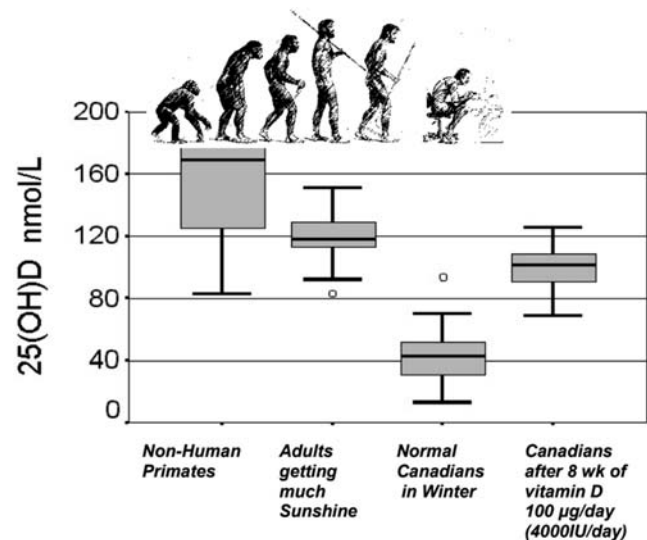
Toronto [44]. The figure begs the question, “Which of those boxplots should be regarded as the best representation of the ‘normal range’ for serum 25(OH)D?” Was there a bias shown on the part of my own laboratory and others when we decided to consider as ‘normal,’ specifically the 25(OH)D results of those persons who were of European Ancestry and who were living outside the tropics?

We should allow ourselves to take the perspective of primate biology. It should be logical to ensure that *H. sapiens*, who are that hairless, tropical, Great Ape, should have serum 25(OH)D levels higher than 80 nmol/L (32 ng/mL), just like what is normal for all other primate species that we care for [45,46]—and just like the 25(OH)D levels of traditionally living in sub-Saharan Africa [42,43]. Ensuring physiologically normal levels of 25(OH)D should be the biologically normal and ethical thing to do [47]. Would any animal-care committee allow any researcher to knowingly keep “healthy” primates in cages so that their serum 25(OH)D levels are less than half of what they would be in the wild?

But instead of thinking about the natural biology of our species, we fixate on what, by modern, clinical laboratory methodology is best described as the “reference interval” for the assay of serum 25(OH)D. Laboratory reference intervals are conventionally defined by the mean plus or minus two standard deviations, i.e., the 95% central interval of the lab test distribution of the local reference group sampled by the laboratory [48,49]. However, serum 25(OH)D is more severely affected by environment and culture than anything else that we can measure in the bloodstream. This metabolite is not like any “normal” laboratory test. Exactly who should make up that “reference group” that defines the reference range for serum 25(OH)D? Basic human biology points to the results published by Luxwolda et al. [42,43] for context as to what should be considered “normal” (Fig. 52.3).

### 3. Nutrition clinical trials cannot match the quality of evidence achieved with drugs

The argument for the status quo in terms of vitamin D starts from the premise that official guidance for vitamin D intake is correct and that avoiding sunshine is the right thing to do. Denial of benefit, beyond official recommendations, for vitamin D and sunshine is the advantaged side of this debate. Any statistical analysis starts off with the default position that the null hypothesis is the correct conclusion. How can you be wrong when you can always sit back and contend that your intellectual standard demands ever more evidence before you can be convinced?



**FIGURE 52.3** Evolutionary perspective of circulating vitamin D nutritional status. Boxes show median and quartile values for 25(OH)D of the primate groups that are represented by the cartoon above. Data are assembled from multiple sources that used the same DiaSorin assay method [5,43–45,50]. Copyright R Vieth.

If only the highest tip of the evidence pyramid will do, then we may as well forget about nutrition altogether. In the context of the role of any nutrient or dietary supplement in the primary prevention of disease, the expectation for the sort of evidence demanded is very high: You need multiple clinical trials that involve a healthy cohort, randomized to a supplementary intake of the nutrient in question. A clinical trial requires an a priori hypothesis that is specified for all to see, at a clinical trials registry such as [www.clinicaltrials.gov](http://www.clinicaltrials.gov). That hypothesis normally specifies that those people who are randomly and blindly assigned to the supplement will end up with fewer of the prespecified disease events than the placebo group.

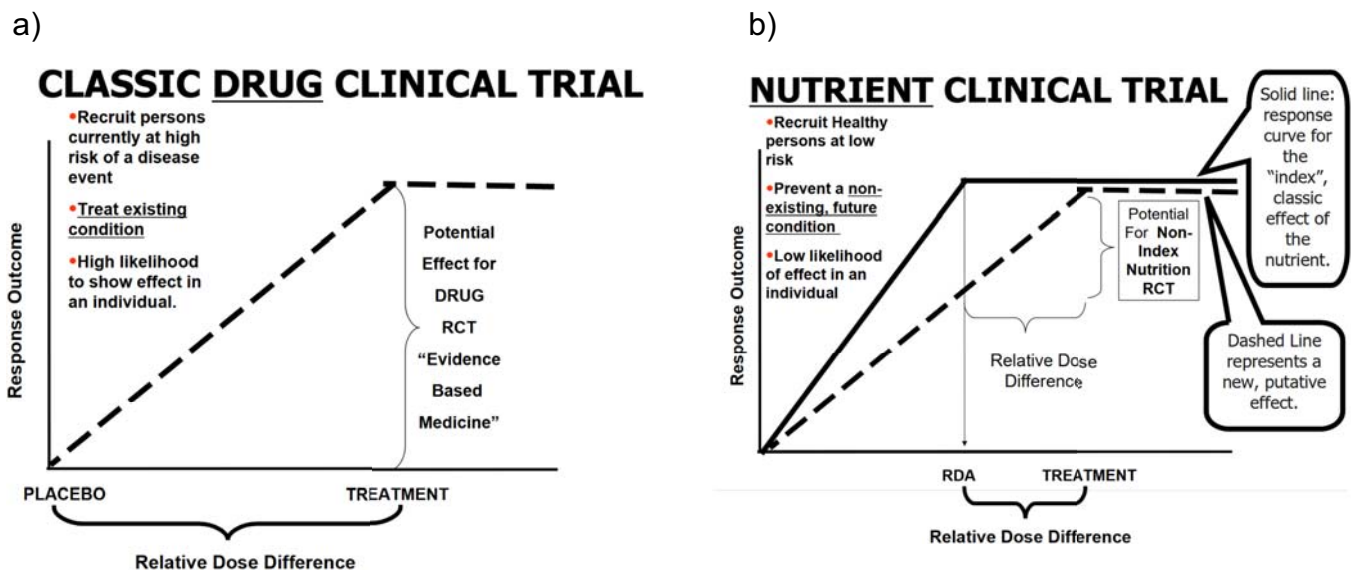
Very few nutrients suit the short-term contexts that can be conducted realistically for clinical trials. One rare exception is supplementation with higher intakes of folic acid, which has been shown to produce fewer occurrences of stroke [51,52]. But the largest of those clinical trials were conducted in China, without America’s folate-fortified flour [53], or they were conducted in subjects with high blood pressure who were already at greater risk of stroke [54,55]. Those shortcomings make the folate clinical trials not quite applicable for all populations. That aspect of research done on a subpopulation is analogous to the way the fracture prevention trials of vitamin D were conducted in the elderly, a particularly susceptible subgroup for fracture outcomes. But for folate, the evidence that a higher intake prevents stroke has not translated to an increase in official dietary recommendations.

“Primary prevention” is the prevention of disease events in healthy persons. “Secondary prevention” is the prevention of further disease events in persons already at greater risk or who were previously affected by the disease. Reviews for the US Preventive Services Task Force have specifically sought out primary-prevention studies of vitamins A, B1, B2, B6, B12, C, D, and E; calcium; iron; zinc; magnesium; niacin; folic acid; carotene; and selenium. The purpose was to review evidence for the benefit and harms of vitamin and mineral supplements in community-dwelling, nutrient-sufficient adults for the primary prevention of cardiovascular disease (CVD) and cancer [56–58]. With the exception of identifying paradoxical harms from beta-carotene and vitamin A with increased all-cause mortality, cardiovascular disease mortality, and lung cancer, the evaluations repeatedly came to the same conclusion that there was insufficient evidence to conclude any benefit or harm of any of the vitamins and minerals for the outcome events of total mortality, cancer, or cardiovascular disease. No nutrient is supported by causal, level 1 evidence for primary disease prevention in healthy adults. “Healthy,” of course meaning that the nutritional status of the study population, is already satisfied in the context of the index condition for each nutrient. (“Index condition” is the classic disease associated with deficiency of the nutrient). Even though these metaanalyses involve vitamin D intakes that range from 400 to 4000 IU per day, they pool vitamin D intake as if it is a binary, yes-or-no thing. The final presentation of the analysis – whether it is vitamin D or any other nutrient – is always that further increasing intake of the nutrient will not

deliver further benefit for the primary prevention of mortality, cancer, or cardiovascular disease.

Most nutrients are thought to exhibit a dose response that reveals threshold intake requirement, whereby the classic deficiency-disease condition for the nutrient is fully prevented at the oral intake typically defined as a recommended dietary intake (RDA) for the nutrient (Fig. 52.1) [2]. Policy-makers assume that intakes beyond that threshold do not provide further benefit, but only raise risk of harm [2,59]. That assumption poses a challenge to prospective clinical-nutrition researchers who are even thinking about conducting research into higher intakes. As innocent as the logic for risk mitigation might appear, it very much hinders the ability to obtain ethics committee approval for research aiming to look into vitamin D intakes higher than the stated upper level (UL) for the nutrient. Recommended nutrition policy becomes like a self-fulfilling prophecy, with research ethics review panels pushing new research in the direction that supports existing recommendations.

In a review addressing the challenges related to evidence-based nutrition, Blumberg et al. pointed out that classic, evidence-based medicine is based on a pharmaceutical model, and that “The level of confidence needed in defining nutrient requirements or dietary recommendations to prevent disease can be different from that needed to make recommendations to treat disease” [60]. Based largely on Heaney’s guidance for the design of nutrient clinical trials [61], Fig. 52.4 shows a comparison between the challenges to obtaining level 1, causal evidence with pharmaceutical clinical trials, versus nutrition clinical trials.



**FIGURE 52.4** Characteristics of clinical trial designs intended to demonstrate therapeutic efficacy of drugs (panel A). Features that pose particular challenges to the design and conduct of primary prevention clinical trial designs for nutrients (panel B). Copyright R Vieth.



The defense for policy-makers is logical: nutrient recommendations require higher confidence than pharmaceutical validation, because nutrient guidance applies to the entire population, and to intakes throughout the lifespan, to prevent (i.e., lower the risk of) primary disease. In contrast, pharmaceuticals are applicable to secondary intervention, that is, once disease exists. The risk/benefit profiles for vitamin D nutrition guidelines are vastly different for primary prevention, versus secondary prevention of disease events.

What further complicates nutritional, primary-prevention-of-disease research is the reality that development of new disease events may take more than the 5-year maximum time frame that is within the realm of what granting agencies are capable of supporting [62]. Table 52.1 lists currently known large randomized trials related to vitamin D. No clinical trials have or will continue with randomized, active-versus-placebo intervention beyond 5 years. However, measurable reductions of disease risk among healthy subjects will almost certainly require longer than that.

“Because the intakes required to prevent many of the long-latency disorders are higher than those required to prevent the respective index diseases, recommendations based solely on preventing the index diseases are no longer biologically defensible” [63,64].

Nutrition-related disorders such as osteoporosis or cancer are obvious examples of long-latency disease consequences, requiring a decade or more to develop or to prevent. If the focus is on causal, level 1 evidence, then we will forever remain blind to the efficacy of any increase in nutrient consumption that is maintained for years.

#### 4. Cancer

It has long been apparent that either sunshine or the vitamin D derived from it can lower cancer mortality [65]. In 2014, a Cochrane systematic review assessed the effect of supplementary intake of vitamin D versus placebo for cancer mortality. Four RCTs involved a total of 44,492 participants [66] with the conclusion was that vitamin D supplementation corresponded to a significantly lower relative risk (RR) for cancer mortality 0.88 (95% CI 0.78–0.98). The mean daily dose of vitamin D was 1146 IU (compared with no supplementation), and the mean length of follow-up was 6.3 years. Since not everyone considered the data convincing, the results of the VITAL study, with its 25,000 participants, were awaited through most of the past decade.

In 2019, the analysis of the VITAL study for the pre-specified primary outcomes was published [67]. The primary outcome of the VITAL study was the “prevention of occurrence of invasive cancer of any type,” and for

that result, the VITAL was not statistically significant [67]. Consequently, Bouillon contends that “No effects of vitamin D supplementation on cancer risk were observed in the large VITAL and ViDA trials” [25]. Likewise, a high-profile editorial by Keaney and Rosen closes cynically with, “Thus, in the absence of additional compelling data, it is prudent to conclude that the strategy of dietary supplementation with either n–3 fatty acids or vitamin D as protection against cardiovascular events or cancer suffers from deteriorating VITAL signs” [68].

The analysis of the VITAL study data regarding an effect of vitamin D did show a lower incidence of advanced (metastatic or fatal) cancer. But that only became clear for the data beyond the 1-year point into the trial [69]. It is important to bear in mind the background population for the VITAL study. The best data regarding general population of the United States is represented by the National Health and Nutrition Examination Survey (NHANES) sample, whose subjects were tested between 2001 and 2006, shortly before the start of VITAL clinical trial. In that NHANES assessment, only 19% of participants had 25(OH)D values  $\geq$  30 ng/mL. That context makes it very likely that the VITAL study, with its relatively high mean baseline 25(OH)D level of 29.3 ng/mL [70], was at least partly affected by a “healthy volunteer selection bias,” similar to what has affected other vitamin D studies [71].

The mean 25(OH)D concentration at baseline of participants in the VITAL study was already at the threshold for a vitamin D nutrition optimum that the Endocrine Society has long advocated [16]. Importantly, the VITAL study participants who were randomized to 2000 IU/day of vitamin D<sub>3</sub> had an average serum 25(OH)D concentration at year 1 that was 40.3 ng/mL (101 nmol/L) [70]. Two points warrant mention here. Firstly, if 2000 IU/day of vitamin D<sub>3</sub> raised the serum 25(OH)D by 11 ng/mL, then how much background vitamin D input must there have been due to sunshine, diet, and supplements in the study subjects to produce their baseline concentration of 29.3 ng/mL? Surely, that baseline supply of the nutrient was already far in excess of any dietary guideline of 800 IU/day or less. Secondly, if development of advanced cancer or cancer mortality over the next 5 years, in healthy adults, is a meaningful objective, then surely, the prevention threshold level for serum 25(OH)D must be at least the 40.3 ng/mL (101 nmol/L). One should also consider that the interquartile range on top of that was 10.4 ng/mL. That totals to 50.7 ng/mL as a reasonable cancer-preventive level for serum 25(OH)D, based on the 75th percentile level of the treatment arm [70].

The VITAL clinical trial needs to be considered as part of a metaanalysis. Similar clinical trials using vitamin D at 2000 IU/day were reported by Lappe et al. albeit in

**TABLE 52.1** Baseline and follow-up 25(OH)D concentrations and vitamin D dosing regimens of selected recent large vitamin D RCTs.

Study acronym or first author	Study population	Baseline 25(OH)D in the entire cohort (ng/mL)	Baseline 25(OH)D in the placebo group (ng/mL)	Follow-up 25(OH)D in the placebo group (ng/mL)	Baseline 25(OH)D in the vitamin D group (ng/mL)	Follow-up 25(OH)D in the vitamin D group (ng/mL)	Vitamin D supplement dose	Study follow-up duration
<b>VITAL</b>	Older general population	30.8 ± 10.0	30.8 ± 10.0	−0.7 from baseline	30.9 ± 10.0	41.8 (mean)	2000 IU per day	5.3 years (median)
<b>ViDA</b>	Older general population	25.3 ± 9.5	24.4 ± 9.6	26.4 ± 11.6	24.4 ± 9.6	54.1 ± 16.0	Initial 200,000 IU, followed by 100,000 IU per month	3.3 years (median)
<b>DO-HEALTH</b>	Older general population	22.4 ± 8.4	22.4 ± 8.5	24.4 (mean)	22.4 ± 8.4	37.6 (mean)	2000 IU per day	2.99 years (median)
<b>D2d</b>	Patients with prediabetes	28.0 ± 10.2	28.2 ± 10.1	28.8 (mean)	27.7 ± 10.2	54.3 (mean)	4000 IU per day	2.5 years (median)
<b>MDIG</b>	Pregnant women	11.0 ± 5.7	11.1 ± 5.5	9.5 ± 5.6	11.0 ± 5.7, 11.5 ± 5.6, 10.8 ± 5.9	27.9 ± 7.8, 40.4 ± 9.4, 44.3 ± 11.2	4200 IU per week, 16,000 IU per week, or 28,000 IU per week	From 17 to 24 weeks of gestation until birth
<b>VIOLET</b>	Critically ill patients	Not reported	11.0 ± 4.7	11.4 ± 5.6	11.2 ± 4.8	46.9 ± 23.2	Single enteral dose of 540,000 IU	90 days
<b>CAPS</b>	Postmenopausal women	32.8 ± 10.5	32.7 (95% CI: 32.1–33.3)	30.9 (95% CI: 30.2–31.6)	33.0 (95% CI: 32.3–33.6)	42.5 (95% CI: 41.7–43.3)	2000 IU plus 1500 mg calcium per day	4 years
<b>Ganmaa</b>	School children	11.9 ± 4.2	11.9 ± 4.2	10.7 ± 5.3	11.9 ± 4.2	31.0 ± 9.1	14,000 IU per week	3 years (median)
<b>EVITA</b>	Patients with heart failure	14.6 ± 6.7	14.1 (10.3–19.7)	16.3 (12.5–23.2)	12.5 (8.6–17.9)	37.2 (25.0–51.4)	4000 IU per day	3 years
<b>Burt</b>	Older general population	31.3 ± 7.8	No placebo group	No placebo group	30.6 ± 8.4, 32.5 ± 8.0, 31.3 ± 7.4	31.0 (mean), 52.9 (mean), 57.8 (mean)	400 IU per day, 4000 IU per day, 10,000 IU per day	3 years

Data are shown as mean ± standard deviation (SD) or as medians with 25th to 75th percentile, if not otherwise indicated; for the MDIG trial only groups with no postpartal intervention are shown.  
 Full citations to the studies listed in Pilz et al. [62] Table used with permission and open source publication.

conjunction with calcium supplement [72,73]. Recent metaanalyses have consistently come to the unambiguous conclusion that although vitamin D supplementation does not affect total cancer incidence, the analyses do unambiguously show that mortality from cancer is lower in the vitamin D-supplemented arms of clinical trials [74–76].

The evidence-based conclusion of benefit from double-blind, placebo-controlled clinical trials of vitamin D<sub>3</sub> is based on the binary, yes-or-no conclusion of statistical significance. To turn that binary knowledge into practice requires a statement of how much vitamin D supplementation is needed. The thing that is probably more important, is what serum 25(OH)D level should people aim for? It is logical to conclude conservatively from the efficacy demonstrated in the VITAL study that sustaining serum 25(OH)D at the 25th percentile of the treatment arm—i.e., 30 ng/mL (75 nmol/L) is a prudent preventive threshold level for healthy adults. Consistent with that value is a cross-sectional analysis of 25(OH)D data from one clinical trial [72] combined with data and a group of self-reported cohort referred to as Grassroots Health [77].

## 5. Mortality

Mortality is the least ambiguous outcome of all. Prospective epidemiological data suggest a protective association of higher serum 25(OH)D levels. Stated more correctly, the data consistently show higher mortality among those classified into the lowest group for serum 25(OH)D. Schottker et al. conducted a metaanalysis of eight prospective cohort studies from sample populations from Europe and the United States. The analysis was done by assembling the data on all the 6685 individual participants of the studies and classifying them into country quintiles of serum 25(OH)D. Mortality was consistently highest for those in the lowest 25(OH)D quartile. The relationship did not differ across countries, sexes, seasons of blood draw, or age groups [78]. Despite differences in mean serum 25(OH)D among reported studies, Schottker et al. observed that it was always the lowest quantile that showed highest mortality, making it difficult to define a threshold serum 25(OH)D for lower mortality [78]. However, there are suggestions of higher mortality for higher serum 25(OH)D quintiles; for example, the Newcastle 85+ Study showed a U-shaped higher mortality relationship [79]. But for the latter cohort, the higher mortality in those at the highest category of serum 25(OH)D was no longer significant after statistical adjustment for mental health and morbidity-related variables. That suggests a “confounding by indication” bias, whereby vitamin supplementation is higher because of known risk of, or presence of,

disease. A similar analysis has been reported for the Chinese Longitudinal Health and Longevity Study (CLHLS). In that study, Mao et al. looked at plasma 25(OH)D levels in 2185 Chinese adults older than 79 years (mean 93 years) [80]. There, all-cause mortality decreased progressively with rising plasma 25(OH)D, with the lowest mortality in those with 25(OH)D at or higher than 75 nmol/L (30 ng/mL). Among 3408 NHANESIII participants, older than 64 years and prospectively followed up for a median of 7.3 years, all-cause mortality was highest in those with baseline 25(OH)D levels less than 50.0 nmol/L, but the authors concluded that levels of at least 100.0 nmol/L may be necessary for better survival [81]. One problem with the available epidemiological data is that very few people have 25(OH)D higher than 75 nmol/L. Sparse numbers of data points result in wide confidence intervals, to the point that some, such as the IOM, interpret the uncertainty of wide confidence bands as indicative of greater risk at higher ranges of 25(OH)D levels [2]. Lastly, Ford analyzed data from 7531 participants in an NHANES cohort in the United States further and reported the fully adjusted HR per 10 nmol/L of vitamin D was 0.93 (95% CI: 0.86–1.01) [82]. I mention the NHANES data here because these epidemiological survey results on mortality are consistent with the metaanalysis of randomized clinical trials reported by Autier and Gandini, as described in the next paragraph.

The key questions remain, “Is mortality lower in healthy subjects who are randomized to higher intake of vitamin D versus placebo? And if so, what is the optimal daily intake recommendation?” The first to address these questions were Autier and Gandini, whose metaanalysis consisted of 18 independent randomized controlled trials, involving 57,311 participants [83]. The doses used in the clinical trials conducted up to the year 2006 ranged from 300 to 2000 IU/day (all were vitamin D<sub>3</sub>). For the pooled vitamin D arm of those trials, the relative risk for mortality from any cause was 0.93 (95% confidence, 0.87–0.99), versus placebo, regardless of whether these osteoporosis clinical trials included calcium supplements. The findings of the Autier and Gandini metaanalysis were confirmed in the report done for the Cochrane Collaboration and authored by Bjelakovic et al. [66]. Several interesting conclusions were drawn by Bjelakovic et al. The clinical trials involving vitamin D<sub>2</sub>, alfalcidol, or calcitriol had no significant effect on mortality [66]. The lack of success in clinical trials using vitamin D<sub>2</sub> should not come as a surprise to anyone who has compared the characteristics of vitamin D<sub>2</sub> versus vitamin D<sub>3</sub> [84–86]. The more recent metaanalysis by Zhang et al. included data from long-awaited, large randomized clinical trials, and there, the conclusion was that vitamin D supplementation alone was not associated with all-cause mortality in

adults compared with placebo or no treatment [74]. What makes the recent, large clinical trials relevant is that subjects were younger and healthier than the subjects were in the osteoporosis-focused trials done earlier. Hence, with the healthy subjects, event rates for death as a proportion of the sample were lower, biasing the results toward a null outcome for overall mortality. Nonetheless, despite no effect on mortality from cardiovascular disease, cerebrovascular disease, or ischemic heart disease, Zhang et al. found that vitamin D supplementation lowered cancer mortality by 16% (95% CI 0.74–0.95) [74].

A comment is warranted regarding the conduct of large clinical trials and the dosage interval. The challenge for anyone designing the trial is, how can one ensure long-term compliance to the protocol of a double-blind, randomized clinical trial in healthy people who, individually, will almost certainly not be able to detect any benefit from taking part? There are three obvious solutions: have salaried research staff regularly engage with study subjects as a means of keeping participants motivated; maximize sample size to overcome confounding factors, dropouts, or imperfections; and lastly, simplify the task of taking the study drug/nutrient to minimize the effort for participants. A longer dosing interval is a well-recognized method of improving compliance, not just for pharmaceuticals but also for vitamin D [87]. The first two approaches make the research too expensive for most granting agencies to fund. The last option, to simplify the ordeal of taking the drug/nutrient, is the easiest approach. However, clinical trials in which vitamin D is taken at intervals of weeks or months [11,88,89] or even a bolus dose of vitamin D annually have usually achieved null, or even adverse effects [90]. Nonetheless, metaanalyses generally combine all data from all available clinical trials, regardless of dosing protocol [2].

It is not enough to deliver a “threshold” level for serum 25(OH)D. Unlike other vitamins, vitamin D is not a metabolic cofactor. Cholesterol, the precursor substrate for steroid hormones, circulates and is available in millimol-per-liter concentrations. Like cholesterol, vitamin D is the structural precursor for a steroid-like hormone. But unlike cholesterol, the vitamin D and 25(OH)D are available to the body in miniscule, nanomolar concentrations; that is, vitamin D metabolite concentrations are six orders of magnitude less than cholesterol, the paradigm for how the rest of endocrinology functions! The point here is that nanomolar concentrations of enzyme substrate add unique complexity to the way the synthesis and breakdown of the hormone, 1,25(OH)<sub>2</sub>D, are regulated. The enzymes of the vitamin D system function under the unusual circumstance of first-order reaction kinetics. Yield of product is determined not just by the amounts of the hydroxylase enzymes but also by substrate concentrations as well

[91]. It takes time for the vitamin D system to adjust to nonphysiological, sudden, bolus doses of vitamin D [92,93]. Since vitamin D is normally acquired gradually through long-term exposure of skin to sunshine, acute bolus doses of vitamin D are not physiological. The dosage interval for vitamin D, taken either as a drug or a supplement, has long been a personal interest of mine. It is particularly upsetting that despite warning against long dosing intervals for vitamin D in 2009 [92], subsequent clinical trials used them, and those negative clinical outcomes have been assimilated into metaanalyses along with trials that used daily dosing. Distinctions to be considered in metaanalyses must include the form of vitamin D, the daily equivalent dosage, and the dosage interval [94].

## 6. Cardiovascular

Nutrients reputed to lower risk of cardiovascular disease include fish oils, antioxidants, selenium, and vitamin D. Much of the basis for having undertaken those studies lies in epidemiology and theory.

Metaanalysis of clinical trials has been highly convincing for the null hypothesis that vitamin D supplementation at various doses has not lived up to the expectation of cardiovascular benefit [51,95]. But then, how can one explain the cross-sectional data pertaining to serum 25(OH)D and cardiovascular health [81,96]? Autier et al. contend that the survival benefits related to higher serum 25(OH)D are secondary to other factors such as diseases that may keep people less active and indoors, as well as lifestyle. They conclude that “associations between 25(OH)D and health disorders reported by investigators of observational studies are not causal” [97].

However, Mendelian randomization analyses do point to a causal connection between serum 25(OH)D and cardiovascular disease risk [98,99]. It is just that the dose response for the outcome of cardiovascular benefit with vitamin D is satisfied once serum 25(OH)D reaches 20 ng/mL (50 nmol/L). In other words, most clinical trials of vitamin D probably resulted in a negative, null-hypothesis result, because they started with a serum 25(OH)D that was already high enough to minimize its role in cardiovascular disease risk. Not all outcomes for vitamin D nutrition need to exhibit the same dose–response curve.

What must not be overlooked in terms of cardiovascular health are the effects that sunlight on the skin per se has on the cardiovascular system, as presented by Liu et al.: Firstly, sunshine warms the skin, which causes vasodilatation and lower blood pressure. Secondly, the skin is a production and storage location for nitric oxide, a potent vasodilator, and which is released into the general circulation when skin is



exposed to sunshine. Thirdly, UVA, corresponding to natural sunlight exposure for 30 min at noon on a sunny day at 41 degrees north latitude, vasodilates the arterial vasculature in a way that is independent of nitric oxide synthase or skin temperature [100]. This complex of mechanisms prompted Liu et al. to conclude “We are concerned that well-meaning advice to reduce the comparatively low numbers of deaths from skin cancer may inadvertently increase the risk of death from far higher prevalent CVD and stroke, and goes against epidemiological data showing that sunlight exposure reduces all-cause and cardiovascular mortality [101].” The cardiovascular and mortality relationships with skin cancer are consistent with the work of Lindqvist et al. who surveyed Swedish women about sun avoidance. Lindqvist et al. showed that women who minimized their exposure to sunshine exhibited rates of cancer and overall mortality that were comparable to effect of smoking cigarettes [102].

## 7. Toxicity

Warnings about higher intakes of vitamin D [25,103] are often disingenuous in their repeated referencing to tolerable upper limitations based on the IOM discussion of risks with vitamin D [2], and where that IOM discussion focused in particular, on the clinical trial of Sanders et al. [90]. That is, despite multiple references to toxicity, objections to higher intakes to vitamin D were all fixated on the same publication, namely Sanders et al. a clinical trial published in a high-profile journal, but in which vitamin D was given as an annual bolus dose. It should not come as a surprise to anyone, if there is harm associated with the once-yearly administration of any nutrient. Nonetheless, the observation of increased rates of falls after the once-a-year administration of vitamin D seems to be taken as a suitable thing to use as basis for a generalization about the risks of vitamin D. I contend that the repeated citing of Sanders et al. in the context of vitamin D safety by the IOM and others [2,103] is done because this report is a convenient way to help convince an uncritical readership about the risks of vitamin D. Years ago, I investigated the basis for statements about vitamin D toxicity in the context of what might be pertinent to dietary guidelines and was surprised that I could not track any of the statements down to the actual published evidence [5]. Hypercalcaemia is the index of vitamin D toxicity, and for that, the field has not changed. However, the more recently characterized feature pertinent to adverse effects with vitamin D relates to the intermittent, bolus dosing described elsewhere in this chapter [92–94].

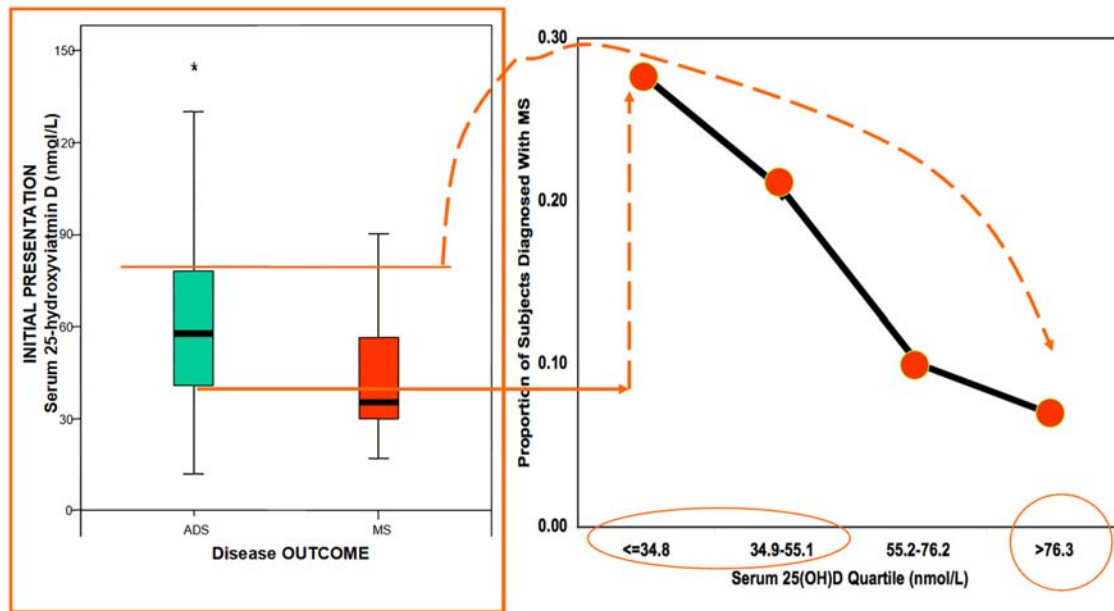
Concern about toxicity of vitamin D that is consumed at 4000 IU/day becomes moot if one considers that this

is within the amount of vitamin D produced naturally from sunshine. That is, the resulting level of serum 25(OH)D, exceeding 30 ng/mL (75 nmol/L), is physiological and normal for the humans [42,43]. Moreover, 48 weeks of vitamin D<sub>3</sub> taken at 14,000 IU daily was safe in a randomized clinical trial patients with multiple sclerosis [104–106]. I mention the multiple sclerosis clinical trials to confirm that the no observed adverse effect level (NOAEL) for vitamin D<sub>3</sub> consumption ranges of up to 10,000 IU/day as specified in earlier reviews and including by the IOM [2,5,107]. The application of a safety margin to that NOAEL results in the “tolerable upper intake level” (UL) that specified by the IOM as 4000 IU/day. I am not advocating vitamin D intakes beyond 4000 IU/day, but rather, restating here some of the known evidence of a wide margin of safety for vitamin D.

## 8. Multiple sclerosis

Chapters 101 and 102 of this book focus on the role of vitamin D in multiple sclerosis, which is an acquired autoimmune disease. For consideration as to a desirable threshold for serum 25(OH)D in children in terms of risk for multiple sclerosis, consider results published by Banwell et al. [108]. Briefly, serum 25(OH)D levels were measured in 302 children at the time when they presented with incident central nervous system demyelination syndrome (usually an isolated, acute phenomenon) at healthcare facilities across Canada. Whether or not those children progressed toward a diagnosis of multiple sclerosis was assessed after a mean follow-up of 3.2 years after presentation. In multivariable models, independent predictors of the eventual development of multiple sclerosis were HLA-DRB1\*15 alleles, remote Epstein–Barr virus infection, and low serum 25(OH)D. There were no interactions between the three predisposing factors. Risk of eventual diagnosis of multiple sclerosis was lowest in those children who, at the time of presentation, had serum 25(OH)D higher than 74 nmol/L (Fig. 52.5).

Is there any causal, level 1 evidence that children or adults who consume additional vitamin D will lower their risk of developing multiple sclerosis? Those who work in the field of Mendelian randomization regard the method as capable of demonstrating a “causal” connection. There are now many publications that used Mendelian randomization and that have sufficient statistical power to relate genetically estimated variations in serum 25(OH)D to risk of multiple sclerosis [99,109,110]. If there is compelling evidence for a causal connection between serum 25(OH)D concentration and risk of multiple sclerosis, the key question remains, exactly what can be done about it?



**FIGURE 52.5** Boxplots showing serum 25(OH)D levels measured on 302 children at the time they presented with acute demyelination syndrome and classified according to whether or not they were diagnosed with multiple sclerosis within the next 3.2 years of followup (panel A). Risk of subsequent diagnosis of multiple sclerosis for each quartile of those 25(OH)D values (panel B). These are graphical presentations of the data from the previously published study by Bantwell et al. [108]. Copyright R Vieth.

In December 2011, the National Multiple Sclerosis Society in the United States hosted a conference entitled “Vitamin D and MS Prevention International Workshop.” The MS Society invited statisticians, experts in clinical trials, multiple sclerosis, and nutrition from around the world to develop a plan. Despite intense discussion about what to do, or how to answer the key question about the role of vitamin D, now, over a decade later, nothing came of it. No registered clinical trial deals with the primary prevention of multiple sclerosis [111]. The reasons for this lack of progress are obvious if one thinks through an example of a power calculation that shows what would be needed for a suitable clinical trial (Fig. 52.6 boxes). It is not realistic to expect that any double-blind, randomized clinical trial will ever appear that overcomes the challenges of funding and conducting suitable primary disease prevention research that requires 1 million person-years of placebo-controlled intervention.

There is now relevant RCT evidence that vitamin D supplementation lowers the risk of the secondary outcome of all incident autoimmune diseases as confirmed by medical record review. Although multiple sclerosis was not included, the subjects in the VITAL study who were randomized to 2000 IU/day of vitamin D<sub>3</sub> were 22% less likely to be diagnosed with an autoimmune disease [112]. It is worth pointing out that same daily vitamin D<sub>3</sub> intake, 2000 IU/day, was the dosage that was the one most considered at the multiple sclerosis prevention conference mentioned in Fig. 52.6. Based on the perspective of Sackett [27], this is a

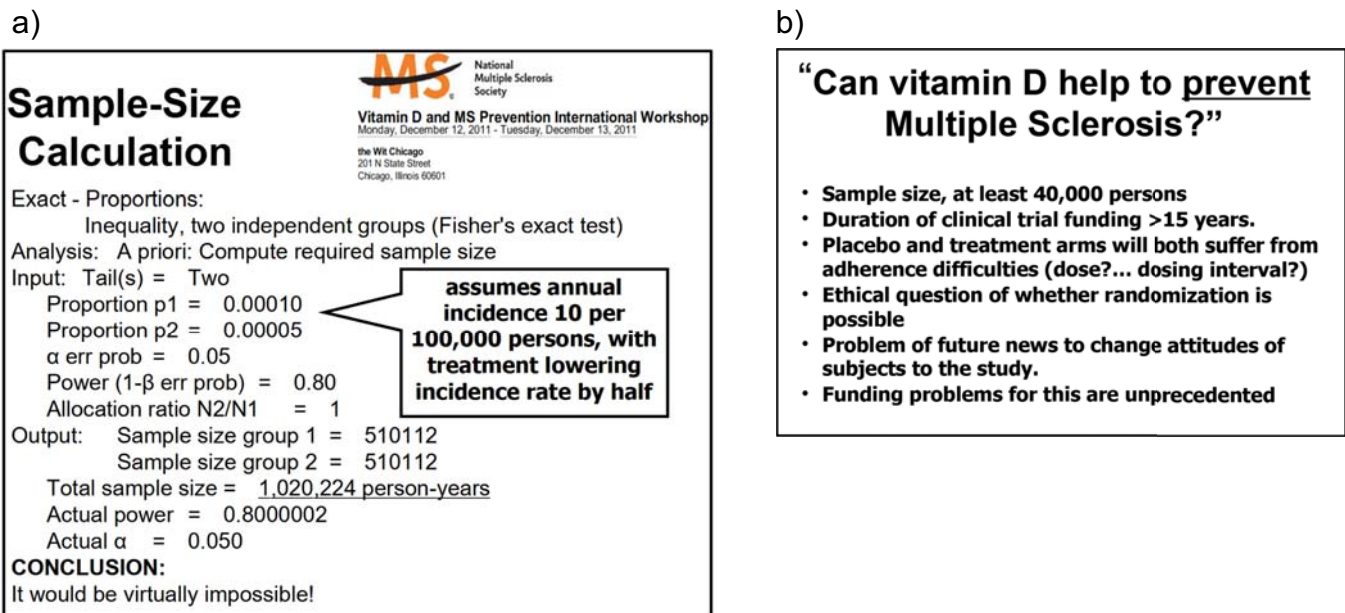
situation that requires advice that is based on the best available evidence, which in this case is something less than a double-blind RCT.

## 9. COVID-19

Before the era of COVID-19, observational studies consistently reported that lower serum 25(OH)D was associated with higher risk of upper and lower acute respiratory infections (ARIs). For example, among a nationally representative sample of 14,108 adults in the United States, who were asked at the time of testing, 4.8% reported having had an acute respiratory infection in the previous 30 days. After adjusting for season, demographic factors, and clinical data, having serum 25(OH)D below 30 ng/mL (<75 nmol/L) was associated with 58% higher odds of acute respiratory infection, and those rates of respiratory infection increased progressively with declining serum 25(OH)D concentrations [113].

Since early 2020, there have been many non-peer-reviewed preprints made available online in addition to properly accepted publications on the topic “COVID, vitamin D.” Manson and Bassuk cited several unpublished studies [114], but several of the more interesting reports that they summarized are no longer available to access, presumably because they were withdrawn, or they failed peer review. Complete knowledge on the topic of COVID-19 will take years. By the time the role of vitamin D in COVID-19 becomes clear, it may not matter any more.





**FIGURE 52.6** Power calculation arising from MS society workshop to address the question, “does vitamin D lower the risk of MS in healthy persons?” (panel A). The implications of that power calculation (panel B).

One of the earliest published reports relating serum 25(OH)D to COVID-19 infection was by Hastie et al. Their manuscript was submitted on April 19, 2020, within the first month of when most of the world was locked down. The manuscript was accepted for publication in record time, the very next day [115]. The investigators used UK Biobank data on 502,624 participants aged 37–73 years from across England, Scotland, and Wales. The serum 25(OH)D level had been measured at the time of recruitment, between the years 2006–2010. Median 25(OH)D had been lower in the Biobank participants who, at least 10 years later, in 2020, had a confirmed COVID-19 infection. In the infected subjects, the 25(OH)D median had been 28.7 nmol/L (IQR 10.0, 43.8) (i.e., median 11.5 ng/mL) compared with “other participants” who had had their median measured at 32.7 (IQR 10.0, 47.2) nmol/L (i.e., median 13.1 ng/mL). In other words, persons with confirmed COVID-19 infection had had significantly lower vitamin D nutritional status than those who were not infected ( $P < .01$ ). However, after statistical adjustment for 15 other variables that included predictors known to affect or be affected by vitamin D status, such as ethnicity, season, and health status, the 4 nmol/L (1.6 ng/mL) difference in serum 25(OH)D between COVID-19 infected versus “other participants” was no longer statistically significant. It is hardly worth asking the question, but should 4 nmol/L (1.6 ng/mL) really be expected to make any difference in people who, a decade prior, had such low 25(OH)D levels? The UK Biobank sample size of hundreds of thousands of people was very impressive. Less impressive was the assumption that a

decade-old result for 25(OH)D should affect risk for COVID-19 now. Comfort of a journal to accept a negative conclusion is evident, because after the authors adjusted for several factors that normally do correlate with serum 25(OH)D, there was no longer a statistical relationship. My point is that statistical nonsignificance was forced inappropriately. Adjustment for factors that are known to determine the independent variable in a relationship will of course neutralize the apparent effect of that variable (i.e., the authors neutralized the ability to detect any effect of the 25(OH)D level). The publication by Hastie et al. lacks evidence of a critical peer review. If there is a publication bias, it is that journals have a “bad news bias,” whereby acceptance of submissions favors negative results involving vitamin D over submissions offering “good news” results.

Another early publication was submitted in late April 2020, but that article showed a positive outcome, and it took longer to review. D’Avolio et al. addressed the question of whether serum 25(OH)D is lower in patients with PCR-confirmed COVID-19 infection compared with those who tested negative [116]. In their COVID-19-positive patients, serum 25(OH)D was (11.1 ng/mL; 27.8 nmol/L), significantly lower than in the comparable patients who tested negative (24.6 ng/mL; 61.5 nmol/L) ( $P = .004$ ). Subsequent similar work, such as that of Dror et al., shows that patients with vitamin D deficiency (defined as  $<20$  ng/mL) were 14 times more likely to have severe or critical disease than patients with 25(OH)D of at least 40 ng/mL (at least 100 nmol/L) (odds ratio [OR], 14; 95% confidence interval [CI], 4 to 51;  $P < .001$ ) [117]. Consider the context of serum

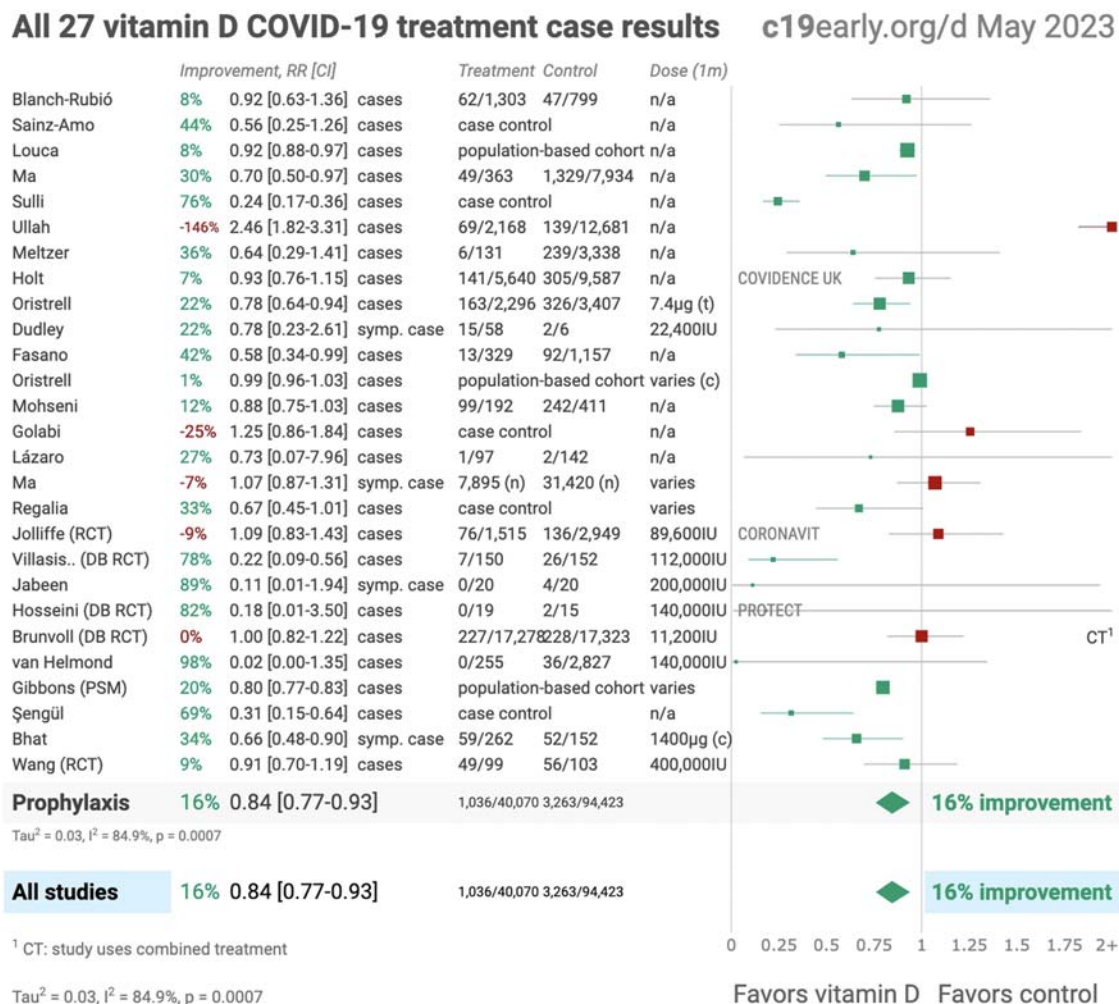
25(OH)D among these studies: Hastie et al. concluded no prognostic differences in serum 25(OH)D for COVID-19 positive patients versus other Biobank subjects, for whom all median values were well below 20 ng/mL (50 ng/mL) [115], while the reports that do show lower serum 25(OH)D concentrations in COVID-19 patients, all groups had levels well above 20 ng/mL (50 ng/mL) [116,117].

Research into COVID-19 has exploded dramatically, but the formal review process has been slow to respond. For example, a Cochrane collaboration produced a “living systematic review” that analyzed data up to March 2021 [118]. Even though the authors promised to update periodically, there is still no update two years later, at the time of this writing. Others, like the National Institutes of Health in the United States, updated their guidance in late 2022, after the severe part of the pandemic was over, and vaccinations were common [119]. Despite the slow and conservative approach of official guidance, some credible academics have been conducting an ongoing, real-time metaanalysis covering

vitamin D and COVID-19 [120]. The website to access their metaanalysis is <https://vdm-meta.com>. The contributors to [vdm-meta.com](https://vdm-meta.com) have chosen to remain anonymous to avoid the emotion, politics, and controversy that has surrounded the entire field of COVID-19. Although [vdm-meta.com](https://vdm-meta.com) is not peer reviewed in the normal sense, the rigor of data collection and level of descriptive analyses of the studies are credible. It merits the attention of anyone interested in any clinical aspect of vitamin D in the COVID-19 epidemic. Fig. 52.7 is an example of the ongoing metaanalysis. The publications cited for the metaanalysis shown in the figure are detailed at the website [120].

Among the challenges that are best described as unavoidable biases to elucidating whether taking more vitamin D can lower risk of undesirable aspects of COVID-19, such as risk of infection, or severity, or hospitalization or death are:

1. COVID-19 continues to evolve into multiple variants, causing changes to the nature of the disease itself, and its efficiency of transmission;



**FIGURE 52.7** Random effects metaanalysis of clinical trials of vitamin D for the outcome of preventing COVID-19 infection. This is an example of the ongoing analysis of vitamin D and COVID-19. Presented at [vdm-meta.com](https://vdm-meta.com) [120], accessed May 23, 2023; Reprinted with open-source permission.

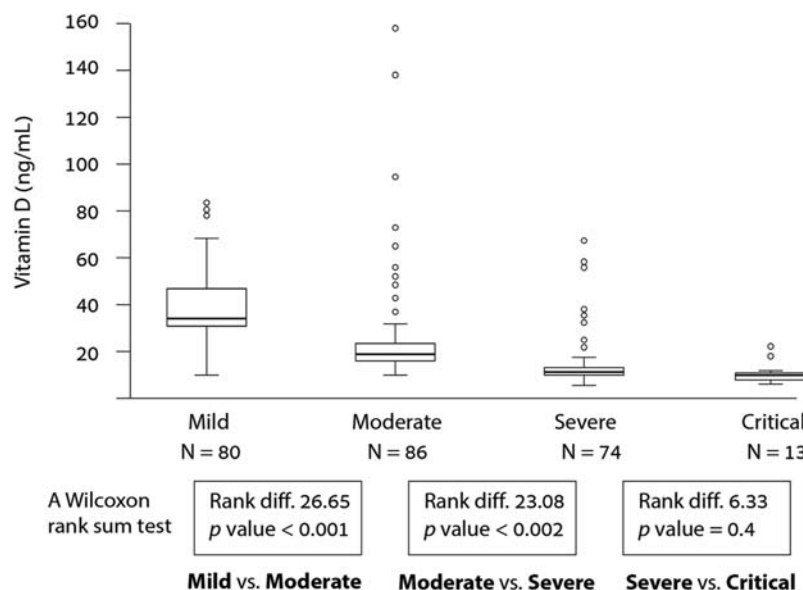
2. human behavior, the wearing of masks and physical distancing change during the pandemic;
3. vaccination lowers incidence of the disease;
4. efficacy of vaccination varies, and it wanes with time.

For COVID-19, can there be any residue of benefit left to detect in a clinical trial of a nutrient like vitamin D? As an example of the ongoing challenges listed before, a preprint of a submitted manuscript shows a well-conducted clinical trial by Jolliffe et al. involving 3100 participants in the United Kingdom [121]. That vitamin D clinical trial shows no benefit from 6 months of 3200 IU vitamin D<sub>3</sub> daily (see Jolliffe, Fig. 52.7). The problem was that by 6.0 months into the clinical trial, to June 2021, 89.1% of participants had received one or more doses of a COVID-19 vaccine <https://www.medrxiv.org/content/10.1101/2022.03.22.22271707v1>). The impressive level of vaccination of the participants casts doubt on validity of this clinical trial and this clinical trial is another example of the “healthy volunteer bias,” which poses challenges that must not be ignored when assessing disease-prevention research for nutrition in general.

Clinical trials that are not confounded by the emergence of vaccines for COVID-19 are rare. However, in April 2022, Villasis-Keever et al. published a double-blind, placebo-controlled clinical trial that was conducted from July to December 2020 in hospital workers who were at high exposure to infected patients [122]. Of the 192 subjects completing the 45-day protocol, SARS-CoV-2 infection rate was lower in those randomized to 4000 IU/day vitamin D<sub>3</sub> than in the placebo-treated group. Infection rates were 6.4% versus

24.5%, respectively, ( $P < .001$ ) (see Villasis in the meta-analyses shown in Fig. 52.7). Baseline 25(OH)D was relatively low, at 18.3 (interquartile range 14.6,22.9) ng/mL (median 46 nmol/L), and the level increased by 8.8 ng/mL with treatment. The RCT published Villasis-Keever is a rare clinical trial that met the challenges of the time: it was conducted relatively early in the pandemic, it was not affected by vaccines, the study population was healthy but did exhibit the expected high rates of infection events because they were front-line medical workers. This study can be classified as “level 1 evidence” for a prophylactic effect with 4000 IU/day of vitamin D<sub>3</sub>.

As an example of opinion-based guidance, a petition published online of recommendations for vitamin D is listed at <https://vitamindforall.org/letter.html>. The page lists the daily personal intakes of many people knowledgeable about vitamin D (including some who are authors of chapters in this book). While not scientifically valid, the listing shows the personal conviction that comes with knowledge about the field of vitamin D. Manson and Bassuk closed their thoughtful “call to action” commentary about vitamin D and COVID-19 with the following words: “during the current pandemic, a supplement containing 1000–2000 IU/day of vitamin D would be reasonable” [114]. However, if one considers the serum 25(OH)D concentrations that accompanied the benefit observed in their VITAL clinical trial [70] and if one looks at the relationship of serum 25(OH)D in patients tested positive COVID-19 versus disease severity (Fig. 52.8), then it does seem rational to advise the public to sustain serum 25(OH)D at levels



**FIGURE 52.8** Severity of COVID-19 infection in relation to serum 25(OH)D measured within 2 years prior to the positive PCR test. Box-and-whisker plots of the most recent preinfection serum 25(OH)D levels ( $N = 253$ ). Shown are comparisons of median 25(OH)D levels between the four categories of COVID-19 disease severity as determined by the WHO definition (WHO/2019-nCoV/clinical/2020.5). Note that 75% (top three quartiles) of those classified with mild COVID19 had serum 25(OH)D that was at least 30 ng/mL (75 nmol/L). Reprinted with open-source permission from [117].



of at the very least 30 ng/mL (75 nmol/L) by whatever means available, be it from sun exposure, diet, or supplement. To ensure that almost all adults have serum 25(OH)D levels that exceed that minimum value requires an input of vitamin D<sub>3</sub> approaching 4000 IU/day for white adults [34]. For adults with dark skin who live at high latitudes, the intake would need to be substantially higher yet [123].

## 10. Conclusion

Evidence presented in chapters throughout this book reveals that vitamin D nutrition holds great potential benefits for health and disease. It is easy to remain skeptical of any benefit beyond current guidelines. The classic justification for skepticism about all biomedical research is exemplified by John Ioannidis, who is well known for his contention that “most published research findings are false” [124]. In contrast, a reading of Sackett [27], who details what evidence-based medicine is and what it is not, would be more appropriate when it comes to the debate about vitamin D. Evidence-based medicine leads to the conclusion that while most research is not perfect, imperfection is not a good reason to ignore the evidence that is at hand. Ignoring evidence for prevention of disease creates unnecessary risk for disease.

## 11. Summary points

- The biologically “normal” range for serum 25(OH)D is best represented by levels observed in traditionally living people in sub-Saharan Africa. Their serum 25(OH)D levels are at least 30 ng/mL (75 nmol/L) with median values of about 40 ng/mL (100 nmol/L).
- Dietary guidelines for vitamin D derive from the historical use of vitamin D in a teaspoon of cod-liver oil, and clinical trials of double that dose that succeeded in preventing fractures in older subgroups.
- Most of the evidence supporting a 25(OH)D threshold of 30 ng/mL (75 nmol/L) comes from prospective epidemiological data, not clinical trials.
- There is no nutrient for which clinical trials have shown level 1 evidence for primary disease prevention in adults younger than the age of retirement. Because it may be impossible to produce clinical trials in nutrition that match the quality standards of pharmaceutical trials, a standard of evidence that is less than “level 1” may be required.
- Vitamin D nutrition, whether from sun exposure, diet (including fortification), or supplementation, benefits musculoskeletal health, autoimmune disease (including multiple sclerosis), cancer mortality, as well as risk of COVID-19 infection, its severity and outcomes.
- To sustain a 30 ng/mL (75 ng/mL) threshold for serum 25(OH)D in 97.5% of the population requires a combined vitamin D<sub>3</sub> supply of 4000 IU/day via UVB exposure, diet, and/or supplement.

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# Methods of evaluating population studies of vitamin D: strengths and weaknesses

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## OBJECTIVES

- Summarize the main goals of population-based studies to our knowledge of vitamin D and health.
- Provide a conceptual framework of the various methods that can be used to assess vitamin D status in epidemiologic studies.
- Summarize the various methods used to assess vitamin D status, including epidemiologic studies, including circulating 25(OH)vitamin D, dietary and supplemental intake, sun exposure, predicted 25(OH) D, and genetic determinants.
- Describe the main challenges inherent in all epidemiologic studies of vitamin D and health.
- Summarize the main strengths and limitations of each of the major study types, including ecologic, cohort, case–control, interventional and Mendelian randomization studies.
- Understand the key issues in interpreting results from various study designs to inform on causal associations between vitamin D and health or disease outcomes.

## 1. Introduction

Population studies can be divided into two main types: observational and interventional (experimental). It is often stated or inferred that observational studies evaluate whether associations exist but cannot prove causality of the associations. Causality can only be

definitively established in interventional studies, typically a double-blinded randomized controlled trial (RCT). While this concept is true in principle, definitive RCTs are not always available or feasible, and even when available, RCTs have their own limitations. Issues related to RCTs are briefly addressed here, but several other chapters in this book will consider this type of study in more detail. Because of the long induction periods over which many diseases develop and progress, and the multifactorial nature of disease, typically no single approach can address all the potentially relevant shortcomings simultaneously. Thus, it is critical to understand the major strengths and limitations of all the approaches utilized to determine whether any association is likely to be causal.

Various types of epidemiologic study designs have been implemented to test the hypothesis that vitamin D is associated with numerous medical conditions and diseases. Meanwhile, only limited interventional data (RCTs) have been conducted for some of the disease end points of interest. This chapter will present an overview of the types of epidemiologic study designs utilized, and their strengths and limitations in addressing various hypotheses related to vitamin D. Relevant examples are given for illustration. This chapter focuses on methodologic issues in designing and interpreting epidemiologic studies and does not summarize in detail specific disease topics, which can be found elsewhere in the book. This chapter can serve as a basis for understanding the methodologic issues that may enhance understanding of these specific vitamin D–disease relationships.

This chapter will be divided into four sections. First, the various methods of assessing vitamin D status in

epidemiologic studies will be reviewed. Secondly, four key general issues in assessing validity of studies will be summarized. Thirdly, the major types of study designs that have been utilized will be reviewed, with major strengths and limitations to take into account. Finally, considerations when synthesizing an entire body of studies on a topic, as opposed to evaluating inference based on an individual study, will be provided in the conclusions.

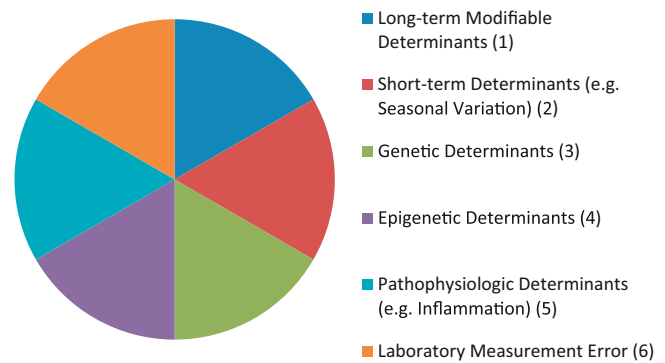
## 2. Methods of assessing vitamin D status

### 2.1 Conceptual framework of assessing long-term vitamin D status

Vitamin D status can be influenced by diet (including supplements and fortification) and by solar or artificial ultraviolet-B (UV-B) light exposure. In addition, vitamin D status is affected by genetic factors and by endogenous factors, such as body mass. The multifactorial determinants of vitamin D status add challenges in designing epidemiologic studies and interpreting results. The gold standard to assess vitamin D status is typically a measure of circulating concentrations of 25-hydroxyvitamin D (25(OH)D). This measure is sometimes feasible and available in population studies, but often other surrogates are used instead. Yet the utility of a single measure of 25(OH)D to estimate long-term 25(OH)D status is not as simple as it may first appear. It is thus illustrative to consider factors that contribute to the variability in 25(OH)D level in a population.

In simple terms, one can think of measuring 25(OH)D in a given population and then examining the variation observed among the individuals. In most cases, there will be distribution of values, perhaps a normal distribution, with some individuals measuring high, some in the middle, and some low. A key question is what are the factors or determinants that ultimately determine whether a measurement is in the high or low range? Fig. 53.1 shows a pie chart of potential determinants of a measure of 25(OH)D. Each of these can contribute to the 25(OH)D level, and from a population perspective, these all can contribute to the variation in 25(OH)D levels. The actual proportions of each of the six factors in explaining variation of 25(OH)D will vary across populations and specific studies. As shown in Fig. 53.1, conceptually, we can think of at least six factors that could contribute to the variation of a single measure of 25(OH)D:

1. long-term modifiable determinants (e.g., vitamin D intake, sun exposure, body mass, region (latitude));
2. short-term determinants (e.g., seasonal variation, recent behaviors);



**FIGURE 53.1** Main sources of individual variation in a single 25(OH)D measure in a given population. The actual proportions will vary by the specific characteristics of the population.

3. genetic determinants;
4. epigenetic determinants;
5. pathophysiologic responses (e.g., systemic inflammation lowering 25(OH)D level);
6. laboratory measurement error.

For chronic diseases for which long-term vitamin D status is etiologically relevant, long-term determinants (1) are more important than short-term exposures (2). For outcomes for which current vitamin D status is important (e.g., acute infectious diseases), short-term exposures such as those due to seasonal (solar) variation may be relevant. Laboratory measurement error adds variation in 25(OH)D levels (6), but these do not reflect long-term status and can be considered as short-term determinants, that is, this error will cause differences in measures from the same individual taken at two separate time points. Measurement error does not reflect long-term actual differences and would generally weaken a true association between 25(OH)D and a disease if one exists.

Genetic determinants (3) are likely to be relevant for long-term status; however, if the genetic factors have biologic effects beyond influencing 25(OH)D levels, the 25(OH)D level determined by genetics may reflect biologic actions not related to vitamin D. Based on genome-wide association studies, only a small proportion of variation has been explained (<5%) [1]. For example, one study identified 69 independent common single nucleotide polymorphisms (SNPs) of genome-wide significant for 25(OH)D that explained 3.1% of the variance in 25(OH)D levels [2]. Although genetic factors appear to explain a relatively low proportion of the total variation in 25(OH)D, this variation is generally not associated with lifestyle factors such as intake or sun exposure behaviors. This lack of correlation between genetic and lifestyle factors can be exploited in Mendelian randomization studies, which can help determine causality of an association because any association observed

is unlikely to be caused by confounding from lifestyle factors (see [Section 4.5](#)).

Epigenetic determinants (4) [3] are likely to be important but currently are poorly understood. For example, it is known that individuals vary considerably in their change in 25(OH)D in response to a given intake of vitamin D, that is, some individuals will have a robust increase, and some will have minimal increase. These differences do not appear to be related to genetic factors primarily, so epigenetic determinants potentially could account for a significant amount of the variation of 25(OH)D levels. For example, in dosing studies of vitamin D on 25(OH)D blood levels, there is generally a wide variation in the individual response with no obvious explanation. One study suggested a potentially important role of epigenetics in determining 25(OH)D levels. Specifically, DNA methylation levels of cytochrome P450 (CYP) enzymes (CYP2R1, CYP24A1, CYP27A1, and CYP27B1) at CpG (cytosine–phosphate–guanine) islands predicted variation in raising 25(OH)D levels after vitamin D supplementation [4]. Epigenetic determinants likely contribute to variation in 25(OH)D levels, but the implications are not straightforward. For example, if infant or childhood deficiency of 25(OH)D results in an epigenetic response to upregulate 25(OH)D conversion, and this is carried over to adulthood, the higher 25(OH)D in adulthood may actually reflect in part deficiency of 25(OH)D earlier in life. Finally, pathophysiologic responses may be operative (5). One hypothesized relationship is that inflammation lowers 25(OH)D levels—thus, low 25(OH)D level may indirectly reflect inflammatory processes. As discussed in the following, this potential could affect inferences related to 25(OH)D level and disease.

Our goal in an epidemiologic study of chronic disease would be to acquire a long-term, time-integrated measure of 25(OH)D, the presumed measure of vitamin D status. Yet, a measure of 25(OH)D, especially a single measure over a course of one's lifetime, while very useful in studying etiology of disease, is a complex measure that is affected by many factors. Some of the factors displayed in [Fig. 53.1](#) may often add noise by being only short-term or cyclical such as seasonality (2) and measurement error (6), cause potential confounding (i.e., an association between low 25(OH)D status and higher risk of a disease may not reflect a causal association, but rather an association with inflammation) (5), or reflect complex relationships as a result of epigenetics (4).

## 2.2 Measures of circulating 25(OH)D

A measurement of circulating 25(OH)D incorporates the many factors that influence 25(OH)D status, both

cholecalciferol production from skin exposure to UV-B radiation and intake. Conceptually, if our goal is to assess cumulative long-term vitamin D status, on the order of decades for cancer, we might take frequent measures of 25(OH)D, say at monthly intervals over 10 years, and we presumably would get an excellent “gold standard” measure by averaging these. However, most studies typically only have access to a single measure of 25(OH)D, or a few at most. Thus, a critical question is how well a single measure would reflect long-term vitamin D status. Fortunately, 25(OH)D levels track over time so, in general, a single measure does reflect, albeit imperfectly, long-term 25(OH)D status. Limited data address the critical issue of how well a single measure is a good indicator of long-term vitamin D status. In one study of middle-aged to elderly US male health professionals, the correlation of two 25(OH)D measures ~3 years apart was 0.7 [5]. In another study of US men and women, the Spearman rank correlation coefficients comparing values at baseline to those taken within 1 year, 1 year apart, and 5 years apart were 0.65, 0.61, and 0.53, respectively [6]. In a Norwegian study, the correlation coefficient between serum 25(OH)D measurements taken at specific intervals from 1994 to 2008 ranged from 0.42 to 0.52 [7].

These correlations are reasonably high to be useful in etiologic studies. Yet, there are also secular trends in overall levels that may affect interpretation of results. For example, in the Canadian Multicentre Osteoporosis Study over a 10-year period starting in 1995–97, serum 25(OH)D increased by 9.3 (7.3–11.4) nmol/L in women and by 3.5 (0.6–6.4) nmol/L in men. Because of this increase, the percentage of participants with 25(OH)D concentrations <50 nmol/L was 29.7% at baseline and 19.8% at year 10 follow-up. Part, but not all of the increase, was due to an increase in vitamin D supplement use over the course of the study [8]. In the United States using national representative data from the National Health and Nutrition Examination Survey, vitamin D supplementation from sources other than multivitamins/multiminerals increased from 5.1% to 19% between 1999 and 2012 [9]. Overall, these findings suggest that 25(OH)D levels correlate reasonably well over time (for example, 5–15 years) for a single measure to provide meaningful results for epidemiologic studies. Nonetheless, there is some degree of measurement error, including some secular trends that can change absolute levels of 25(OH)D, which would tend to attenuate any real association.

A specific complexity in studies of 25(OH)D is that levels fluctuate throughout the year due to seasonal variances in sun exposure, so cases and controls are often matched for season of blood draw. One study provides a good example of adjusting for seasonal variation by regressing 25(OH)D concentrations in control



participants on week of blood draw using sine–cosine functions based on fluctuations of 25(OH)D throughout the year [10]. While matching or controlling for season reduces some extraneous variation in 25(OH)D level, it cannot overcome some potential limitations in a single measure. For example, if the nadir of 25(OH)D level in winter month is etiologically most relevant for a particular outcome, the samples that were collected in non-winter months may be less informative or even uninformative.

An additional important issue regards complexities in 25(OH)D measures using different assays and even within batches using the same assay. Having consistent reliable measures is critical in the formation of recommendations regarding absolute levels of 25(OH)D and disease risk. Technical issues related to measuring 25(OH)D are discussed in [Chapters 48–50](#). Based on available studies, some caution should be used when considering the absolute levels of 25(OH)D. Nonetheless, if cases and controls are matched and assayed in the same batch, then the relative ranking is valid if the assay has high interbatch reliability. Thus, studies to date are still useful in examining for differences in 25(OH)D levels between cases and noncases for hypothesis testing whether 25(OH)D levels are associated with a disease. In analyses that pool data from various studies, calibration of the various assays has proved useful [10]. However, some caution is required before establishing the actual quantitative dose–response relationship. Standardization of 25(OH)D tests in the future could ameliorate this issue. Additional issues regarding measures of “free” or “bioavailable” 25(OH)D due to the vitamin D binding protein are becoming more recognized and have been considered in a small number of epidemiologic studies to date. These may, or may not, become important in future epidemiologic studies, but are not considered in detail here, but can be referred to in [Chapter 7](#) of this book.

## 2.3 Measures of vitamin D intake

Vitamin D is relatively scarce in the common foods consumed by most populations. Moreover, not all populations fortify foods with vitamin D, and even when fortified, the amounts are relatively limited. For example, a glass of fortified milk (in the United States) contains only 100 IU vitamin D, a level that is small compared with amounts of vitamin D that can be formed on exposure to UV-B radiation [11,12]. In most populations, probably more vitamin D is made from sun exposure than is ingested through diet, though in some populations at high latitudes, vitamin D from marine sources may be the dominant sources. Thus, vitamin D intake may be an important contributor to 25(OH)D levels, especially

in winter months in regions at high latitudes [13]. During these time periods, intake may be the sole source, contributing vitamin D to the depleting sources of 25(OH)D accumulated in the summer and autumn. One important consideration of studies of vitamin D intake is that, depending on the specific population of interest, intake of vitamin D may be predominantly from one or a few sources, such as fatty fish, fortified milk or margarine, or supplements. Thus, dietary vitamin D intake will tend to be highly correlated with other dietary factors (e.g., omega-3 fatty acids in fish, calcium in milk, and other vitamins and minerals in supplements). If the correlation is very high (e.g., correlation coefficient >0.7), separating the influence of vitamin D from the correlated factor may be difficult from a statistical perspective.

An additional consideration is that ergocalciferol (D2) is often used in supplements, and ergocalciferol has been estimated to be less potent than cholecalciferol (D3) in raising 25(OH)D level [14]. Most databases do not take into account whether the source of vitamin D in supplements is from D2 or D3. In the past decade in the United States, the vitamin D dose available from supplements (including cholecalciferol) has increased and prevalence of use has increased. This change may increase the opportunity to conduct observational studies that can examine associations based on supplemental vitamin D intakes at higher levels.

## 2.4 Measures of sun exposure as a surrogate of vitamin D status

Because the major source of vitamin D is from sun exposure, some studies have used presumed sun exposure as a surrogate of vitamin D status. In some cases, especially in ecologic studies, solar UV-B based on factors such as latitude and cloud cover is used as the surrogate of vitamin D status on a population-wide basis. In some case–control and cohort studies, region and some questionnaire-based measures of sun exposure have been the measures used to assess potential vitamin D status on an individual basis. Some surrogates that have been used, such as sunburns or actinic damage from sun exposure, may represent relatively few extreme acute exposures to sun rather than frequent exposures that may be more relevant for vitamin D synthesis. Measurement error and possibly recall bias in case–control studies (discussed in the following) in assessing past exposures are issues that may potentially influence validity. The validity of using sun exposure as a surrogate of vitamin D status is strengthened if there is some evidence supporting that the surrogate used actually correlates with 25(OH)D level in the study setting.



Some objective methods to assess sun exposure, such as the use of reflectometry, have been utilized. For example, one case–control study of advanced prostate cancer was based on use of a reflectometer to measure overall sun exposure [15]. This method assesses the difference between facultative skin pigmentation on the forehead (a sun-exposed site) and constitutive pigmentation on the upper underarm (a sun-protected site). Then the difference between facultative and constitutive pigmentation is used to estimate sun exposure. In this study, sun exposure estimated by reflectometry was inversely associated with risk of advanced prostate cancer. Because these approaches do not directly quantify vitamin D status, they may offer support that individuals with higher potential exposure to UV-B that generates vitamin D are associated with an outcome, but they do not quantify the dose–response relationship between vitamin D status and disease risk. Most importantly, sun exposure could affect a disease by mechanisms other than production of vitamin D. For example, it has been hypothesized that solar ultraviolet (UV) radiation releases nitric oxide (NO) from storage forms in the skin, which can lower blood pressure [16]. If true, 25(OH)D as a measure of sun exposure could be associated with lower blood pressure, but this may not be a causal association.

## 2.5 Measures of predicted 25(OH)D levels

An additional approach to assess vitamin D status is to use known predictors of 25(OH)D level based on data on the individual level to formulate a predicted 25(OH)D score. The use of multiple determinants should provide a better estimate of 25(OH)D status than using a single determinant such as diet or region of residence. In one example of this approach, the predicted 25(OH)D score was formed and then examined in association with risk of colorectal cancer in men of the Health Professionals Follow-Up Study [17]. First, in a sample of 1095 men, actual plasma 25(OH)D level was the dependent variable in a multiple linear regression. The independent (predictor) variables were geographical region, race as a surrogate of skin pigmentation, dietary intake, supplement intake, body mass index, and leisure time physical activity, a surrogate of potential exposure to sunlight UV-B. Based on the regression coefficients, a score was calculated for each of ~47,000 cohort members who had information on these variables. This variable was then examined in relation to subsequent risk of incident colorectal cancer cases. In the multivariate analysis, a 25 nmol/L increment in 25(OH)D was associated with a 37% reduced risk of colorectal cancer. This association persisted after controlling for body mass index and physical activity.

The predicted 25(OH)D approach may have some advantages and disadvantages compared with the use of a

single measurement of circulating 25(OH)D in epidemiologic studies. The measurement of 25(OH)D is more direct and intuitive and incorporates some of the sources of variability of 25(OH)D not taken into account by the score. However, the predicted 25(OH)D measure may provide a reasonable measure of vitamin D status over a long period of time because some factors accounted for by the predicted 25(OH)D score are immutable (for example, skin color) or relatively stable (region of residence, body mass index), and these can be updated periodically (e.g., repeated dietary measures). In addition, a blood measure is prone to certain biases (see 4 and 5 in Fig. 53.1) discussed in the following and measurement error (Fig. 53.1, 7). Thus, although a predicted 25(OH)D will not capture all of the variation in circulating 25(OH)D, much of the extra variation in a measure of circulating 25(OH)D may actually contribute to noise and bias.

To assess the accuracy of the predicted 25(OH)D, the main method used is to compare the R-square of the model of predicted to actual 25(OH)D. Some of the reported R-squares (i.e., percentage of variation explained of a single 25(OH)D measure) have ranged from around 15% to 47% [18–21]. When compared with a single 25(OH)D level, the R-square for these “surrogates” have been typically considerably less than 50%, leading some to question the utility of this approach. Yet, the R-square may not be the best criterion to evaluate the potential usefulness of the predicted 25(OH)D score in epidemiologic studies. The argument that an R-square statistic reliably assesses validity assumes that a single measure of 25(OH)D is a perfect assessment of long-term 25(OH)D status, such as over a 10- or 20-year period. However, as summarized before, correlations between measures of 25(OH)D over a period of 3–10 years can be as low as 0.4 to 0.5. For a true “gold standard,” this correlation should be 1, or at least very close to it. Incorrectly, when considering the correlation between the actual measurement of 25(OH)D and the predicted 25(OH)D, all of the measurement error is assumed implicitly to be in the predicted score, but the inherent measurement error in a single 25(OH)D assessment to estimate long-term 25(OH)D status will dampen the correlation between the 25(OH)D measurement and the predicted score. Thus, the R-square between the 25(OH)D measurement and the predicted score should not be simply used to assess the ability of the predicted 25(OH)D measurement to estimate long-term 25(OH)D status.

A more useful criterion in evaluating the predicted 25(OH)D score to test a vitamin D–disease hypothesis is to consider the difference in long-term 25(OH)D tested by “high” and “low” exposure from the predicted 25(OH)D. Consider the example of an RCT of vitamin D and a chronic disease outcome. Two criteria we might

consider is (1) the increment in 25(OH)D caused by the study dose of vitamin D and (2) the time duration of the study. For example, a relevant question could be “does increasing 25(OH)D by 10 ng/mL for 10 years affect incidence of colorectal cancer?” Typically, RCTs of vitamin D present the mean 25(OH)D level in the treated group and the reference group, rather than the R-square of how well the treatment explains total variation in 25(OH)D status. The difference in the mean actual 25(OH)D levels in those with high versus low predicted scores incorporates both real variation in the population and measurement error from the questionnaire. For example, in three cohorts, the differences in mean actual 25(OH)D level between extreme deciles of predicted 25(OH)D score ranged from 9 to 12 ng/mL [22]. In actual cohort analyses with disease end point, to compute the predicted 25(OH)D score, repeated measures on diet and supplements every 2–4 years over a 30 year period can be incorporated. Notably, use of vitamin D supplements increased dramatically over time in the cohort; unlike a single measure of 25(OH)D, multiple questionnaire measures were able to account for the individual fluctuations of dietary and supplementary intakes of vitamin D (as well as other factors) over a 30-year period. In the Harvard cohorts, predicted 25(OH)D has provided comparable results to measured 25(OH)D for colorectal, pancreatic, and endometrial cancers [23–25]. Some have proposed using predicted 25(OH)D based on such data as region, sun exposure habit, as a screening tool for vitamin D deficiency [26].

## 2.6 Measure of genetic variation of circulating 25(OH)D

Genetic factors that affect 25(OH)D concentrations have been identified. For example, genetic variants around 7-dehydrocholesterol reductase (DHCR7) (rs7944926 and rs11234027) and CYP2R1 (rs10741657 and rs12794714) influence 25(OH)D levels either through synthesis of previtamin D from 7-dehydrocholesterol in the skin or through conversion of vitamin D to 25(OH)D in the liver [2]. These are relatively small determinants of 25(OH)D among all the determinants and explain only a small proportion of variation (<5%) in 25(OH)D concentrations. Yet, in adequately large study populations, statistically significant differences in mean 25(OH)D will be observed in individuals depending on their genetic variants. Furthermore, a genetic score can be formed based on multiple variants, with individuals possessing more of the variants associated with high 25(OH)D status having highest average 25(OH)D levels and those with fewest such variants with lowest average 25(OH)D. From an epidemiologic perspective, an advantage of the

variation in 25(OH)D due to genetics is that this will tend to be not associated or only weakly correlated with the nongenetic determinants of 25(OH)D (Fig. 53.1). This fact can be exploited in studies termed Mendelian Randomization studies, which examine the genetic association of 25(OH)D determinants with disease risk. These are discussed in Section 4.5).

## 3. Main challenges in the epidemiologic study of vitamin D and disease

There are numerous challenges in discerning causal associations in epidemiologic studies. While all these concerns are pertinent to studies in general, some are specifically relevant for studies of vitamin D. In particular, vitamin D status can be assessed in multiple ways, as discussed in this section, and how vitamin D status is assessed may have inherent advantages and limitations depending on the study question at hand. Four main criteria that need to be considered in assessing study validity are introduced in this section. These are each discussed in detail for each type of study design for the assessment of vitamin D status. This does not represent a list of all factors affecting validity in epidemiologic studies, but some of the major issues that should be considered specifically for epidemiologic studies of vitamin D are as follows:

### 3.1 The time period for the assessment of vitamin D status covered should be based on the pathogenesis of the disease end point of interest

Some effects of vitamin D, for example, on rapidly responding physiologic parameters such as blood pressure, could potentially, though not necessarily, become apparent relatively immediately as in days or weeks. In contrast, the effect on some exposures on multistage diseases that involve cumulative processes, such as cancer, may require assessments based on a longer time frame, such as years or even decades. The etiologically relevant time frame when vitamin D may be acting on the disease is critically important to consider. This point is often ignored. The time factor may be especially relevant for situations where early life exposure may influence risk of the disease in adulthood, or for diseases where there may be a long-time lag between the exposure and the appearance of the outcome. For example, it is becoming apparent that adolescence and early adulthood is a period where the risk of adult breast cancer [27] and prostate cancer [28] is especially prone to exposures. For multiple sclerosis, increasing data indicate that exposure to sun in childhood and adolescence (perhaps a

surrogate of vitamin D exposure) is associated with reduced risk later in life [29–31]. In many cases, the etiologically relevant time period is not known. In this case, “null” studies need to be interpreted conservatively as a true effect may have been missed by the study.

### 3.2 Studies ideally should assess the dose–response relationship

From a study of a given disease end point, it might not be adequate to conclude simply that higher vitamin D levels are beneficial. Rather, the dose–response relation should be estimated, which requires precise measures of vitamin D status. The probability of detecting an association in a study (assuming an association exists) depends on the range of vitamin D exposure in that population. This point is seen clearly in an RCT setting. In an RCT, the effect of an intervention may depend on the underlying vitamin D status of the population. For example, if nearly everyone in the study population is sufficient at baseline, no effect of the vitamin D intervention may be seen; if most people are vitamin D deficient at baseline, an effect may be observed if the intervention raises 25(OH)D levels to a therapeutic level. A special feature of vitamin D is that status is determined by dietary intake, supplement use, and sun exposure, and by endogenous factors such as obesity that influence 25(OH)D level. Some studies only measure one aspect of vitamin D status—for example, dietary intake or sun exposure measures or surrogates of sun exposure. While such studies may provide useful information to test the general hypothesis of interest, they do not assess the full dose–response relationship because only some of the determinants of vitamin D status are being addressed. Results from two studies may apparently conflict because the range of 25(OH)D assessed in the two studies was different, and the studies may have captured different ranges of the dose–response relation.

### 3.3 Biases should be reduced or eliminated to the extent possible

Bias refers to some mechanism in the data acquisition process that yields spurious associations in the study population. For example, let us assume that there is no association (neither causal nor noncausal) between dietary vitamin D intake and disease X in a population. If dietary data are collected with reasonable precision, then we would expect to see no association between vitamin D intake and disease X. However, in a case–control study, prediagnostic dietary data are inferred from retrospectively recalled dietary data; thus, it is possible that case and controls recall diets differently.

If so, a bias may occur. For example, more cases may contemplate on their past diet more extensively and thus are more likely to report higher dietary intakes than controls. This tendency can be considered recall bias. Of note, in a prospective cohort study, where diet information is collected before knowledge of disease outcome, recall bias is less likely. Of course, dietary information may not be recalled perfectly; however, because individuals do not know years in advance whether they will develop a disease or not, the errors of recall should be nondifferential relative to the future disease status. Such errors tend to produce nondifferential misclassification, adding “noise” to the measure, which would tend to obscure any associations that may exist. However, if an association is observed, it is unlikely to be caused by recall bias.

### 3.4 Confounding should be limited to the extent possible

The term “confounding” is often used nonspecifically as bias, but among epidemiologists, it has a precise meaning. Confounding occurs (1) when a causal factor for disease other than the one of primary interest exists in the population, and (2) this factor is statistically correlated with the primary factor of interest. This correlation between the factor of interest (e.g., vitamin D) and the other risk factor for disease produces a noncausal (spurious) association between the factor of interest and the disease. In the case of confounding, the information in a population may be collected accurately, but a noncausal association would still exist. For example, if physically active individuals are more likely to have higher 25(OH)D levels, then physical activity could be a potentially confounding factor for the vitamin D–disease association. If accurate information on physical activity were collected in the study, and proper statistical adjustments made, then the observed association between 25(OH)D and disease could be eliminated in the multivariable model adjusted for physical activity. Confounding can only be definitively excluded in an RCT, where the prevalence of all other factors is balanced between the treated and the placebo group. In observational studies, judgment must be made on the likelihood of confounding. In well-conducted prospective cohort studies, where biases can largely be excluded, confounding is the paramount factor to consider for a valid result, that is, an observed association is causal.

Fig. 53.1 provides a useful basis to consider the potential sources of confounding in studies of vitamin D. For a factor to exhibit confounding, it needs to have a statistical association with vitamin D status, and to be a risk factor for the disease of interest. The first group of potential confounders to consider are those factors that have a

direct relationship with 25(OH)D (1). For example, obesity is associated with lower 25(OH)D levels, and physical activity, which may increase opportunities for sun exposure, tends to be associated with higher 25(OH)D. These two factors, thus, need generally to be considered as potential confounders because they are risk factors for many diseases. If they are very strong risk factors for a specific disease, such as for type 2 diabetes mellitus, then the possibility of confounding is heightened. Some factors may not directly determine vitamin D status but could be confounders through correlated behaviors. For example, those who consume vitamin D supplements and thereby have higher 25(OH)D levels are also probably more likely to take other supplements (e.g., calcium or a multivitamin). Although these factors may not necessarily directly influence vitamin D status, because they have a statistical association with vitamin D through correlated behaviors, they are potential confounders if they are associated with the disease under study.

Short-term determinants of 25(OH)D (Fig. 53.1, 2) are generally not confounders, but rather add noise (extraneous variation) to any association with vitamin D and thus tend to cause underestimation of a true association. These errors should generally not cause a spurious association to arise if there is no true association.

Genetic determinants of 25(OH)D (Fig. 53.1, 3) are potentially useful in avoiding confounding because they tend not to be associated with many of the determinants of 25(OH)D, especially the ones related to behaviors. Thus, the association between a genetic determinant of 25(OH)D status and a disease will generally not be confounded by determinants such as physical activity, diet, or obesity. However, if a genetic variant utilized to determine 25(OH)D status has nonvitamin D functions in other pathways that are associated with the disease, this can cause a type of “genetic confounding” termed pleiotropy. The approach of using genetic determinants as a measure of vitamin D status has been termed Mendelian randomization and is discussed in the following.

Epigenetic determinants (Fig. 53.1, 4) are potential confounders, at least in principle, though this area is poorly understood. For example, if factor X early in life causes an upregulation of genes that convert vitamin D to 25(OH)D, then factor X could be a confounder if it is an independent determinant of the disease. As a theoretical illustrative example, assume poor vitamin D status early in life (e.g., low intake or low sun exposure) causes an upregulation of genes that enhance 25(OH) production through epigenetic mechanisms, and this upregulation is maintained later in life. This would cause higher 25(OH)D levels to be measured later in life; yet, paradoxically, this contribution to higher adult 25(OH)D may reflect a vitamin D deficiency early in life. Until we better understand what epigenetic determinants of

25(OH)D, it is not obvious how likely these are to be confounders.

Finally, a potentially important confounding factor is whether a pathophysiologic process, such as systemic inflammation, lowers 25(OH)D. In fact, this bias has been offered as a potential explanation of many of the associations observed between lower 25(OH)D levels and many chronic diseases [32]. The argument is that vitamin D would not be a causal factor for the disease, but rather low 25(OH)D levels would merely be a marker of chronic inflammation, the presumed causal factor. While plausible, this mechanism has not received extensive study. In one study, the inverse association between 25(OH)D level and risk of colorectal cancer in two cohorts persisted when the association was further adjusted for some markers of systemic inflammation [33]. This result provides some reassurance that the association between low 25(OH)D and colorectal cancer risk is not entirely due to low 25(OH)D reflecting an inflammatory state. In addition, complementary to the results for plasma 25(OH)D are studies based on predicted 25(OH)D, which show a similar association between low predicted 25(OH)D and colorectal cancer, but are not prone to this particular bias because 25(OH)D is not directly measured [16]. This topic deserves more study. A complicating factor is that some of the potential beneficial effects of vitamin D may be through a reduction of inflammation, so the directionality of the 25(OH)D—inflammatory marker direction requires better understanding [34].

#### 4. Summary of study designs of vitamin D

We consider five major types of study designs. These will be referred to as ecologic studies, cohort studies, case-control studies, randomized interventional studies, and Mendelian randomization studies. The nested case-control study, which is often utilized in studies of circulating 25(OH)D and disease, can technically be considered a case-control study, but because these are typically nested in prospective cohort studies, the issues related to validity are more similar to cohort studies than traditional case-control studies. Thus, nested case-control studies are considered a subset of cohort studies. The major issues affecting validity, summarized in *Main Challenges in the Epidemiologic Study of Vitamin D and Disease* (Section 3), are addressed specifically for each study design.

##### 4.1 Ecologic studies

Ecologic studies are based on examining rates of a disease in an entire population, rather than on an individual basis, and then to correlate some factor of interest based on the population average to the disease rates across



populations. For example, disease rates could be determined within countries, and then intercountry disease rates can be compared. The population can also be based on regions (e.g., states, provinces) within a country. In the context of vitamin D, the comparison that is inferred is typically the different average UV-B exposure as a surrogate of vitamin D status in the population. For example, building on work from Garland and Garland on colon cancer [35], Grant demonstrated that regional solar UV-B radiation inferred by region taking into account factors such as latitude, altitude, and cloud cover correlated inversely with mortality rates of numerous cancers, particularly digestive organ cancers [36]. Although rarely utilized, in principle the population average vitamin D status (e.g., 25(OH)D measurements taken on a representative sample of the entire population) can be used rather than surrogates. Studies of secular trends of a disease within a population are also possible. For example, disease rates following a population-wide intervention such as the implementation of vitamin D fortification can be examined. Insights and limitations from these ecologic studies are summarized here.

One potentially important advantage of ecologic studies is that if there is no substantial migration within the population, the exposure is lifelong. For example, those who have always lived in a sunny environment would have a lifelong potential for more vitamin D production than those who always lived in a less sunny region. If the time-relevant exposure for the disease of interest is early in life (childhood, adolescence), yet the disease typically occurs in middle or older ages, an association can possibly be captured in ecologic studies of geographical residence. In contrast, most serum-based cohorts are composed of middle-aged individuals, so the estimate of 25(OH)D will not reflect early life exposure, and an association for this example could be missed. In cohort and case-control studies, early life geographical residence could be used as a surrogate of past sun exposures and presumably vitamin D status at points earlier in life, at least based on region of residence. For some diseases, these earlier time periods could be most relevant. Ecologic studies are also feasibly done and can be utilized in many settings, providing a broad set of data.

On the downside, region is not a perfect surrogate of vitamin D status and does not take into account individuals' sun seeking or sun avoidance behaviors. Some populations may receive abundant sun but due to cultural or individual preferences may avoid the sun or wear clothing that blocks the sun. The main limitation for some ecologic studies may be the difficulty in controlling for various potential confounding factors. Some population-wide data can be used as covariables, such as average smoking rates, average body mass index, and average alcohol consumption. However, it is not clear if confounding can be adequately controlled

for in this manner because these data are not collected on an individual basis and data sources may vary across diverse populations. Large differences across many factors in populations, for example, in highly economically developed versus less developed countries, may be very difficult to take into account. There may be systematic differences across countries that confound the data. For example, in cancer studies, different rates of screening and detection of cancer across countries could influence apparent cancer incidence rates.

## 4.2 Cohort studies

The three main types of observational studies that assess exposure on an individual basis are cohort studies, case-control, and nested case-control studies. These are often referred to as analytic epidemiologic studies. In cohort studies, a study population is defined, and then vitamin D status is assessed on the individuals, who are then followed over time, often years, or decades, for occurrence of the disease end point of interest. The determination of disease outcome can be based on various methods, such as recontacting the participants for further information or linking to databases (e.g., cancer registries). Then rates of the disease can be compared, for example, in those who are deficient in vitamin D compared with those sufficient in vitamin D. Because vitamin D status is not randomized in the study population, but rather reflects factors such as diet, sun exposure behaviors, and genetics, statistical adjustments must typically be made to account for disease risk factors that could differ in the population by vitamin D status. These risk factors could cause confounding of the apparent vitamin D-disease association, which was discussed in more detail before. Because exposure information is collected before the disease occurs in cohort studies, these studies are often termed prospective studies or prospective cohort studies.

Vitamin D exposure can be assessed in various ways, as discussed in *Methods of Assessing Vitamin D Status* (Section 2). Typical methods are a circulating measure of 25(OH)D, or surrogates such as predicted 25(OH)D, dietary intake or sun exposure. Because information is collected prospectively in relation to disease occurrence, the cohort design can avoid many of the potential biases that may limit case-control studies. To avert many of the potential biases, the follow-up response rate of a cohort study should be high, for example, >90%. Because many biases can be avoided through use of the cohort design when the design and execution are strong, the main consideration for validity involves the assessment of potential for confounding. As discussed in *Main Challenges in the Epidemiologic Study of Vitamin D and Disease* (Section 3), it is critically important to



judge whether an association is causal or is confounded by another factor. When there is a null or weak result, consideration should be given to whether there was considerable measurement error of vitamin D, the range of vitamin D studied was within that of the dose–response relation, and whether the etiologically relevant time period was assessed. These factors would tend to obscure any relationship, rather than to generate a spurious association if none existed.

A nested case–control study has features both of a prospective cohort study and a case–control study. This study design is commonly used in biomarker studies, including that of circulating 25(OH)D. Unlike a typical case–control study, the study population is well defined, and all participants are identified from this population. Typically, the study population is based on an established cohort study, such as the Nurses’ Health Study, where participants have provided a blood specimen, which is then archived under stable, low-temperature conditions [37]. At the end of a defined follow-up period, such as 10 years, all cases of the outcome (disease) of interest are identified. For controls, a random sample is then selected from all the non-cases. When cases and the selected controls are identified, the blood samples are retrieved and assays (e.g., plasma 25(OH)D) are conducted. The data are analyzed according to a case–control design. The nested case–control design avoids certain biases such as selection bias and biases that may arise if the data are collected postdiagnostically. The trade-off versus a conventional cohort study is a small loss in statistical power, but the advantage is an enormous costs savings. In most cases, issues of biases in nested case–control studies should be considered similar as in cohort studies. Because the sample was taken before the diagnosis of cancer, it is unlikely that any association observed results spuriously from the cancer influencing the blood level (frequently called “reverse causation”). Reverse causation could occur in retrospective studies if the cancer itself or treatments cause metabolic changes that affect the 25(OH)D level, or if cancer leads to changes in behaviors (e.g., sun exposure reduced due to treatment or disability) that influence 25(OH)D levels. Thus, studies that have been based on the measurement of 25(OH)D in individuals already diagnosed with cancer need to be interpreted very cautiously because of the potential for the phenomenon of reverse causation. Even when 25(OH)D is measured prospectively, it is advisable to perform a lagged analysis excluding the first several years of follow-up to limit the reverse causation effect of undiagnosed latent disease.

### 4.3 Case–control studies

Case–control studies are studies in which individuals with the disease or end point of interest (cases)

are identified and an appropriate control group is identified. The exposure information (e.g., vitamin D status) is then assessed, typically retrospectively, in both the disease group and the control group. Conceptually, the control group should be a sample derived from all non-diseased individuals who, had they become cases, would have been included in the study as cases. For example, if a case–control study is defined as all cases of colorectal cancer diagnosed in New York City in 2000–04, the controls would be derived from all residents of New York City in this time period who, had they developed colorectal cancer, would have been included in the case set. Information of past vitamin D exposure is then assessed and compared in the case group and the control group. A case–control study can be efficient in the sense that one just needs a random sample of all residents for the study to be valid, at least in terms of selection. If a comparable cohort study were to be done in this example, all of the residents in New York City would have had to have vitamin D status assessed in 2004 and then all followed for colorectal cancer up to 2004. Such a study may not be feasible or prohibitively expensive. Case–control studies can be especially helpful in studying rare diseases because the underlying population in a cohort study would have to be very large to generate adequate case numbers.

In terms of issues of measurement of vitamin D (Issues 1 and 2 in *Methods of Assessing Vitamin D Status, Section 2*) and of confounding (Issue 4), the same considerations exist for case–control as for cohort studies. In addition, bias (Issue 3) is of particular concern in case–control studies [38]. We can consider two main types of potential biases, selection bias and information bias. Selection bias refers to biases in selection of proper controls. As described before, the control group should represent a random sample of individuals who, had they become cases during the study period, would have been identified as cases. In the aforementioned example of all residents from New York City, a practical means of identifying controls would be to use telephone numbers of all New York City residents as the population source. However, it is likely that some behaviors would relate to likelihood that a potential study participant if contacted would respond and agree to participate. If the response rate is low, then bias may occur. For example, it is possible that “health conscious” individuals who take a vitamin D supplement would be more likely to respond than those who do not. The study could then give biased results by making it appear that those who did not develop the disease were enriched with vitamin D supplement users. As response rates have tended to come down in population-based case–control studies in recent years [39], the potential for selection bias has increased. Other methods of selecting controls, such as the use of those hospitalized for other

reasons (than the disease of interest) presumably not related to vitamin D, can be utilized, though it is difficult to conclude that these conditions are not related to vitamin D. If they are, a bias would occur.

Information bias may occur when there is systematic error in the assessment of study information. The main concern is recall bias, which may occur because in a case–control study, information is collected after the case status is already known. If the information is based on recall, for example, of the typical diet 5–10 years before diagnosis, not only may there be error in general in both cases and controls (nondifferential error), but also there may be some differential error where cases have the tendency to over- or underreport intake. This type of bias can sometimes generate a spurious association. For example, cases may think more extensively over their past diet and thus may be less likely to forget food items. If the study involves a nonblinded interviewer, the interviewer may have unconscious biases in eliciting information because he or she is aware of the study question.

Objective biomarker studies are not a panacea for case–control studies, and perhaps can even be more prone for bias. If a blood sample to measure 25(OH)D is taken after diagnosis, changes in 25(OH)D may have occurred because of the disease. In this case, postdiagnostic 25(OH)D status is not an appropriate surrogate for prediagnostic 25(OH)D, the presumed etiologic factor. Some empirical data support that 25(OH)D level may decrease after a major disease diagnosis. For example, in breast cancer, very strong inverse associations between 25(OH)D and risk of breast cancer are observed in case–control studies, in contrast to prospective nested case–control studies, where little or much weaker associations are observed [40]. Some have offered the counterargument that as breast cancer may develop rapidly, case–control studies that measure 25(OH)D closer to the time of diagnosis (albeit after diagnosis) may capture the etiologic relevant time period better than cohort studies with a measure that can be years before the diagnosis [41,42]. If one is strictly interested in genetic factors (see Mendelian randomization studies in the following), case–control studies may provide valid results because genetic status would not change due to the diagnosis.

#### 4.4 Interventional studies (randomized controlled trials)

Intervention or experimental studies are those designed so that the investigator assigns vitamin D exposure, rather than just observing vitamin D status that occurred naturally in the population. A double-blinded, placebo-controlled, randomized intervention

(RCT) is widely accepted as the “gold standard” in establishing a causal association. In this study design, vitamin D status is conferred to the subject randomly by the investigator. The investigator and the subject are blinded to vitamin D assignment until the data analysis stage to prevent any bias. If randomization is successful, typically ensured by a sufficiently large study size, then all other known and unknown determinants of the disease should be equally distributed and uncorrelated to vitamin D status. If an association between vitamin D and outcome is observed, and chance ruled out by a sufficiently large study, then one may infer that the association between vitamin D and the end point is causal. Any other factors that may have contributed to risk of the disease should have been balanced equally in frequency between the vitamin D assigned group and the placebo group. Thus, the vitamin D is the only factor that could have contributed to the difference in disease rates in the treated and placebo groups.

While deceptively simple in concept, “definitive” RCTs are difficult to execute [43]. Because of the expense of performing RCTs of vitamin D and many outcomes of interest, randomized studies are rare for some end points. Although considered the gold standard in addressing causality, in practice, RCTs have a number of practical limitations. The selection of the effective dose may be problematic. Unlike as for a drug, everyone has an underlying baseline level of 25(OH)D, and thus the potential for benefit likely varies among the subjects; those with adequate vitamin D may not benefit from the study intervention and may contribute to null results [44]. For cardiovascular disease, both observational studies of circulating 25(OH)D [45] and dose–response Mendelian randomization studies suggest low 25(OH)D is associated with increased risk only at very low levels, such as below 25 nmol/L [46]. A benefit of vitamin D supplementation has not been confirmed in RCTs, but it is possible that most of the subjects enrolled had levels exceeding the level where any benefit plateaued. For example, in the VITamin D and Omega-3 Trial (VITAL), the mean baseline 25(OH)D level was about 75 nmol/L, so it is unlikely that many subjects had sufficiently low levels to experience a benefit [47].

In addition, there may be poor compliance and contamination by the placebo group adopting the change (for example, taking vitamin D supplements outside of the study protocol). In some cases, the necessary duration for the study may not be adequate to show an effect for a cancer outcome. Finally, the period of time during the long natural history of cancer where vitamin D action is relevant is unknown. For example, in autoimmune diseases such as multiple sclerosis, it may be relatively feasible to design an RCT to examine if vitamin D

reduces progression of the disease, but it is extremely difficult to demonstrate if vitamin D deficiency in infancy and childhood predispose to multiple sclerosis risk in adulthood.

When a well-designed and effectively executed RCT elicits a positive finding, this result may provide strong or even compelling evidence of support for the hypothesis. The largest primary prevention RCT to date is VITAL, a United States nationwide, randomized, placebo-controlled,  $2 \times 2$  factorial trial of vitamin D<sub>3</sub> (cholecalciferol, 2000 IU/day) and marine omega-3 fatty acids (1 g/day) for the prevention of cancer and cardiovascular disease and other major outcomes [47–49]. Another example is the D-Health Trial, which was initiated to determine if supplementing an older population with high monthly doses of vitamin D (60,000 IU of cholecalciferol) can prevent cancer and premature mortality [63]. This trial differs from VITAL in providing infrequent bolus dosing in contrast to daily dosing. Because of the limitations enumerated before, when these studies show a null association, caution must be given not to have undue confidence in the results. Besides correctly reflecting the absence of a true association, one or more of the limitations mentioned before could produce a null association in an RCT.

An important consideration in RCTs that is not relevant for most other types of evidence is that some trials have used bolus dosing. The degree of bolus dosing can vary; for example, some trials have used doses of cholecalciferol ranging from 20,000 IU/week to 500,000 IU/year. In fact, evidence from RCTs suggests that large bolus dosing of vitamin D may have minimal or possibly adverse effects on various outcomes, including rickets, falls, fractures, and respiratory infections [50]. Although bolus dosing is usually effective in increasing 25(OH)D levels, vitamin D (cholecalciferol) is rapidly cleared from the circulation and becomes undetectable in the circulation after about 1 week. Hollis and Wagner have argued that many cell types possess 25 hydroxylase and  $1\alpha$ -hydroxylase activities and are thus capable of converting vitamin D to the active metabolites [51]. This autocrine production of 25(OH)D precludes the requirement of vitamin D hydroxylations in the liver and kidney [11]. Furthermore, because of its low binding affinity to vitamin D-binding protein, vitamin D diffuses into cells more readily than 25(OH)D, which is bound tightly to the vitamin D-binding protein. Furthermore, large-bolus dosing activates the enzyme 24-hydroxylase (CYP24A1), which results in catabolism of 1,25(OH)<sub>2</sub>D [50]. It is plausible that maintaining daily high levels of vitamin D is physiologically superior to infrequent large-dose bolus, even if the same level of circulating 25(OH)D is achieved.

## 4.5 Mendelian randomization studies

A genetic approach to study causation in epidemiologic studies is based on the concept of “Mendelian randomization.” These studies are discussed in more detail in Chapter 62 of this book. In recent years, Mendelian randomization has been applied to the study of nutrition and diseases, including vitamin D [52]. The basic premise of Mendelian randomization is that a person’s genetic variation is generally not correlated to nongenetic cancer risk factors. Thus, if the relationship of 25(OH)D and a disease endpoint were to be causal, an association would be expected between the genetic variants (e.g., single nucleotide polymorphisms or SNPs) that predict levels of 25(OH)D and cancer risk. Mendelian randomization is a useful approach, but assumptions must be clearly understood and evaluated if feasible. The first assumption is a gene–environment equivalence assumption, that is, the genetic effect is equivalent to a comparable environmental effect. For example, a 10 nmol/L increment in 25(OH)D caused by supplementation would yield a similar result on an outcome as a 10 nmol/L difference in individuals resulting from genetic variation. Based on formal instrumental variable analysis, there are three important assumptions. The “relevance” assumption requires that the genetic variants that are being used as an instrument variable are robustly associated with the exposure; the “independence” or “exchangeability” assumption requires that no common causes of the genetic variants and outcome exist (examples include population substructure, assortative mating and dynastic effects); and the “exclusion restriction” assumption requires no independent pathway exists between the genetic variants and outcome other than through the exposure, also known as horizontal pleiotropy. If these requirements are met, Mendelian randomization can provide independent evidence for an association and would suggest that association is causal. Although spurious associations could occur in Mendelian randomization studies, the potential biases and confounding are unlikely to be similar as in conventional observational studies (e.g., cohort studies), so a convergence of results by different approaches increases the likelihood of causality.

The identified genetic variants for 25(OH)D explain a small amount of variation (<5%) of 25(OH)D levels, so the predicted reduction in risk would be small and perhaps undetectable in some studies. Exceptionally large studies are usually required for adequate power. Furthermore, because some of the SNPs associated with 25(OH)D levels also influence vitamin D binding protein concentration and the affinity for 25(OH)D, these SNPs also alter 25(OH)D bioavailability (free

hormone) independently of total 25(OH)D concentration potentially invalidating the “independence” or “exchangeability” Mendelian randomization assumption. For example, possession of the DBP2-encoding variant (rs4588\*A) is associated with lower 25(OH)D levels, but some studies suggest that this DBP2 isoform also has the lowest binding affinity to 25(OH)D, leading to higher levels of free 25(OH)D [53,54]. This, perhaps this variant could lead to both lower total 25(OH)D levels and potentially higher free 25(OH)D levels. If free 25(OH)D is the causal factor for an endpoint, the inverse relationship between total and free 25(OH)D could complicate inferences from studies based on the genetic variation of total 25(OH)D.

Mendelian randomization has been utilized in vitamin D studies for various endpoints. One very large study based in 326,409 UK Biobank Europeans examined the causal relations between 25(OH)D and 106 diseases/traits based on Mendelian randomization analysis using 143 genome-wide significant 25(OH)D-associated variants [55]. The analyses from this study supported a potential causal role of 25(OH)D in increasing height and preventing ovarian cancer, multiple sclerosis, leg fracture, and femur fracture. One study examined 95,766 Danish white participants from three cohorts who were genotyped for genetic variants in *DHCR7* and *CYP2R1* [56], genes affecting plasma 25(OH)D concentrations through synthesis. Plasma 25(OH)D was also measured. Lower measured 25(OH)D levels were associated with an increased risk of all-cause mortality, cardiovascular mortality, cancer mortality, and other-cause mortality. Lower genetically determined 25(OH)D levels were associated with an increased risk of all-cause mortality, cancer mortality, and other cause mortality, but were not associated with lower risk of cardiovascular mortality. These results from this Mendelian randomization study support the observational results of an association between lower 25(OH)D and all-cause mortality, cancer mortality, and other mortality but not for cardiovascular mortality. A broad protective effect on cancer incidence (except ovarian cancer) has not been demonstrated by Mendelian randomization studies [57]. Mendelian randomization studies provide robust support for genetically low 25(OH)D and multiple sclerosis [55,58–60]. These data suggest that Mendelian randomization studies may contribute to the study of vitamin D and disease.

## 5. Conclusions

Population-based association studies are an important component of the overall body of evidence for a given association. The overall synthesis of evidence for an association should include the evaluation of studies

that provide mechanistic insights, including in vitro investigations, animal studies, and human experimental studies on intermediate factors for the end point of interest. Mechanistic studies are important for ascribing causality to observed associations. However, the complexity and multifactorial nature of chronic diseases that occur over many years or decades in free-living populations often preclude simple extrapolations. Even if a causal association is assumed, the dose–response relationship and the etiologic relevant time period when the association may be actively influencing disease risk may not be known. Intermediate markers of a disease may include hormonal, metabolic, immunological, and epigenetic responses, but how these intermediates ultimately relate to a chronic disease may not always be a simple matter because diseases are multifactorial and may develop in stages over many years or decades. Laboratory studies in cell culture or animals permit hypothesis testing under controlled conditions to a greater degree than is feasible in free-living human populations, but results may not be directly generalizable to humans. Population-based studies provide critical information for dose–response, temporality of an association, and for identifying susceptible subgroups of individuals.

Causality of an association can be best established in RCTs, but the utility of RCTs may depend on the specifics of the outcome of interest. RCTs are most useful in testing hypotheses for common end points or a physiologic parameter (such as blood pressure) for which a short-term effect may be predicted. The numbers required may be manageable, and the time required is short enough to ensure high compliance. Susceptible groups (e.g., those with low baseline 25(OH)D who may be most likely to benefit) may be selected. In such cases, RCTs could feasibly generate definitive results. Some large randomized studies conducted over the past decade (e.g., VITAL, D HEalth study) are beginning to yield important information for chronic diseases [47]. PMID: 30886934. Typical intervention times for such RCTs are approximately 5 years.

Intervention periods of 5 years are reasonably long, but important associations could still be missed. Some diseases may have periods of susceptibility and long-time lags to observe an effect. For example, RCTs of aspirin and colorectal cancer only show a benefit emerging after about 10 years from the onset of the intervention [61]. Another example is breast cancer, where the period of breast growth in adolescence is a particularly critical time for exposures to exert effects [62]. RCTs are unlikely to provide definitive data for the hypothesis that vitamin D status during this time influences breast cancer risk. Even standard epidemiologic studies of circulating 25(OH)D assessed in middle or older age would be inadequate in testing the hypothesis that early-life exposure to vitamin D influences breast



cancer risk. Mendelian randomization studies might be useful as the genetic “exposure” should presumably be lifelong and encompass early-life effects.

Often, judgments must be based primarily on observational data, along with supporting mechanistic data. This chapter summarized five different methods to assess vitamin D status, four major considerations in studies to assess validity, and five major types of study designs. These criteria are useful in assessing the likelihood of a study providing a valid result, that is, if an association is observed, whether it is causal rather than reflecting bias or confounding. The use of a biologic measurement of 25(OH)D has been useful in epidemiologic studies, but caution is required in interpretation. As summarized in Fig. 53.1, there are multiple determinants in the variation of 25(OH)D, and some would not reflect a causal association. In fact, if 25(OH)D level were influenced by a process such as inflammation, the potential for confounding (“reverse causation”) would be serious. Complementary studies of vitamin D intake, sun exposure and predicted 25(OH)D would be useful because, while they have their own limitations, they would not be prone to this same bias.

A more recent research approach with substantial potential is Mendelian randomization. A limitation is that only a small proportion of 25(OH)D is explained by genetic factors involved in determining 25(OH)D status, so very large studies are required. When they yield positive results, these studies add to the argument of causality because confounding from lifestyle determinants of 25(OH)D is unlikely to be significant. Mendelian randomization studies can be complementary but would not inform on the dose–response relationship or on the time period of action because presumably the genetic “dose” would extend across the lifespan and one could not tell when vitamin D actions are relevant. That the genetic “exposure” is lifelong is useful in detecting an association, but if one is observed, it may be difficult to tell when in the life course the exposure is most etiologically relevant.

Many lines of evidence contribute to the establishment of a causal association. Because different approaches would have different strengths and limitations, consistency of findings across many different study designs would strengthen the argument for causality. Consistency across populations would also be important. For example, if an association with vitamin D is observed both in populations where most vitamin D is from sun exposure or and in populations where dietary sources of vitamin D are most important, this finding would tend to argue against a single confounding factor explaining the associations. While appropriately conducted RCTs may be required to establish a causal association, all available evidence should be incorporated into determining the relevant timing in the life course, the dose–response, and susceptible populations.

## 6. Summary points

- Randomized controlled trials are considered the highest level of evidence to establish a causal association between vitamin D and various health and disease outcomes.
- Observational studies remain important and complementary to randomized trials, as they can examine associations in very large numbers, for long durations and for wide ranges in vitamin D status, in susceptible subgroups, and can help establish dose–response relationships.
- There are multiple ways to assess vitamin D status, including direct measures of circulating 25(OH)D, dietary and supplementary intake, sun exposure as a surrogate, genetic determinants (Mendelian randomization), and a combination of predictors of vitamin D status.
- It is critical to consider the specific strengths and limitations of the various methods to assess vitamin D.
- Observational study designs include ecologic studies, cohort (prospective) studies, case–control studies, and Mendelian randomization studies. Each has specific strengths and limitations to when considering the potential for bias and confounding to provide invalid results. In general, cohort studies based on measures of circulating 25(OH)D are considered the strongest type of observational studies.
- The best conclusion is made when results from different study designs, which tend to have different sources of biases, converge on similar results.

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## Further reading

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# Worldwide vitamin D status

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## OBJECTIVES

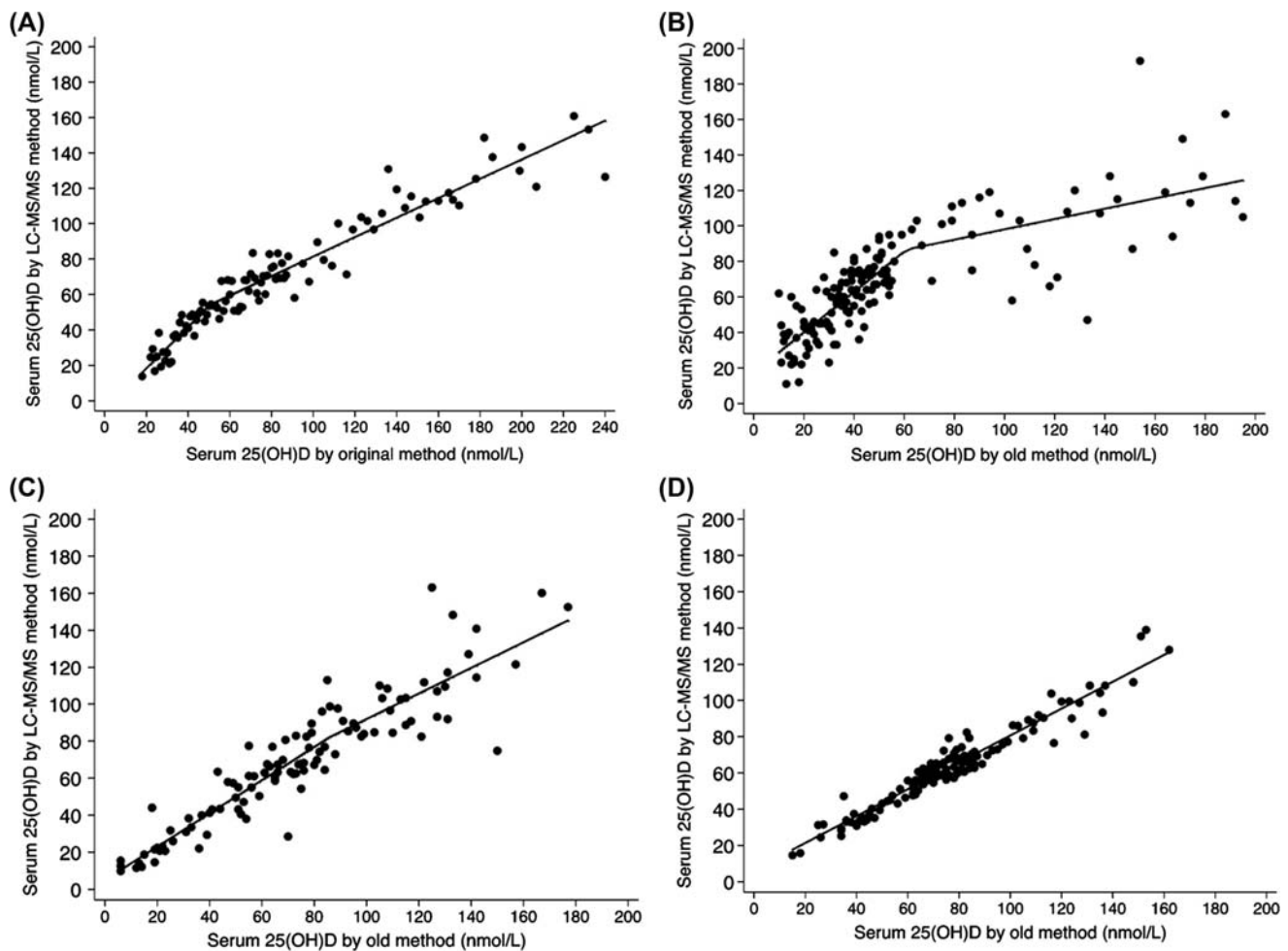
- After reading the chapter, the reader should have insight in the assessment of vitamin D status and the necessary quality control, the worldwide vitamin D status, and the prevalence of vitamin D deficiency and insufficiency throughout continents and countries.
- The reader will also be able to identify vitamin D deficiency high-risk groups.
- The chapter will also describe the influence of nutrition, ethnicity and migration on vitamin D deficiency.
- The chapter will also detail trends in vitamin D status during the last decades, and the implications of the findings.

## 1. Introduction

Vitamin D status has been determined in numerous studies covering all continents and many countries. The measurement of serum concentrations of 25-hydroxyvitamin D (25(OH)D) is used to assess vitamin D status, to diagnose vitamin D deficiency, and to determine the effect of vitamin D supplementation. There is not yet consensus on the required serum 25(OH)D concentration for optimal health. However, most clinicians agree that clinical vitamin D deficiency only occurs when serum 25(OH)D is lower than 25 nmol/L (10 ng/mL) [1,2]. According to the Institute of Medicine,

vitamin D deficiency occurs when the serum concentration 25(OH)D concentration is below 30 nmol/L [3]. Opinions differ on whether the required serum 25(OH)D for optimal bone mineral density, bone turnover, muscle strength, and nonclassical effects should be above 50 nmol/L, 75 nmol/L, or higher [4,5] (differing opinions are discussed in Chapter 51 and Chapter 52).

Another related problem, when comparing vitamin D status between countries, results from assay differences between various studies [6]. These differences may result in bias of 25% between 25(OH)D measurements resulting in large differences in the segment of the population that should be treated for inadequate vitamin D status [7]. Over the past decade, efforts have been made to standardize 25(OH)D assays. Liquid chromatography, followed by tandem mass spectrometry (LC-MS/MS), has become the method of choice due to its better precision compared with immune-based methods (see Chapter 48). The Vitamin D External Quality Assessment Scheme (DEQAS) distributes sera to more than 1000 laboratories and provides the results as the difference from the overall mean value [8]. The Vitamin D Standardization Program (VDSP) uses standards provided by the National Institute of Standardization Technology to improve the accuracy of the assays [9,10] (see Chapter 49). This facilitates comparison between current surveys of vitamin D (25(OH)D) status (Fig. 54.1). However, epidemiological studies done in the past can also be standardized when frozen samples are available. This enables unbiased comparison between different countries and different studies as carried out for the food-based solutions for optimal vitamin D nutrition and health through the life cycle (ODIN) study [11].



**FIGURE 54.1** Standardization according to Vitamin D Standardization Program (VDSP) protocol. The relationship between serum 25(OH)D in a subsample of the Health 2011 (A), Oslo Health (B), Health 2006 (C), and VitmaD (D) samples measured by original 25(OH)D assay and standardized LC-MS/MS at University College Cork. Ref. [10].

Vitamin D status depends on the available amount of ultraviolet light in the sunlight, which varies with latitude and season, on actual sunlight exposure and on skin pigmentation, and the use of sunscreen and clothing (see [Chapter 3](#)). The latter also varies depending on cultural and religious background. The lower the actual sun exposure, the more nutrition becomes important, especially the consumption of oily fish, vitamin D–fortified foods, and vitamin D supplements (see [Chapter 55](#)). In the following paragraphs, vitamin D status and the occurrence of vitamin D insufficiency and deficiency will be discussed in different continents, North America, South America, Europe, Middle East, Asia, Africa, and Oceania. Vitamin D deficiency will be defined as serum 25(OH)D < 25 nmol/L, unless stated otherwise. The number of surveys on vitamin D status is ever growing, and excellent reviews have been published in recent years [12,13]. In the current review, we have included a selection of studies published in the past decade, i.e., from 2011 onward. Subsequently,

studies on vitamin D status in one or more continents performed in a central laboratory will be presented. In these studies, interlaboratory variation in serum 25(OH)D assays does not play a role. In the next sections, ethnic issues and nutrition will be discussed as far as these have consequences for vitamin D status in different countries and continents. In addition, risk groups will be identified and discussed. In the final section, the consequences, i.e., the part of the population that is vitamin D deficient or vitamin D insufficient will be discussed, and finally, conclusions will be drawn.

## 2. Assessment of vitamin D status

Vitamin D status is usually assessed by measuring 25(OH)D in plasma or serum. The assay methods can be divided in immunological methods, including radioimmunoassay and chemiluminescent immunological assay, and chromatographic techniques, including



high-performance liquid chromatography (HPLC) and LC-MS/MS [14] (see Chapter 48). The chromatographic methods have a lower variation and are less subject to bias than the immunological methods. Currently, LC-MS/MS is considered the gold standard. DEQAS follows more than 1000 laboratories worldwide by sending samples, collecting the results, and plotting the values around the general mean [14]. With the VDSP, recent and older population studies within Europe have been cross-calibrated and standardized in the ODIN study [11].

### 3. Study selection

Many reviews on vitamin D status throughout continents or worldwide have been published in the past decade [12,13,15,16]. For this chapter, our aim was to give an overview of the vitamin D status worldwide without attempting to be complete. A search was done in PubMed using the following search strategy: (“2016/07/01”[Date—Publication]: “2022/01/10”[Date—Publication]), and (“Vitamin D”[Mesh] “Vitamin D deficiency”[Mesh]) and (“prevalence”[tw] “population”[tw]), specified according to continents. Studies between 2011 and 2016 were selected from the former edition of this chapter. We sought for studies preferably with more than 1000 subjects, population-based studies rather than convenience samples or biobank studies. When more studies were available for one country, we selected studies with standardized assessment of 25(OH)D or those that used chromatographic (preferably LC-MS) instead of immunological methods. Many regions in the world just recently started with vitamin D assessment in their population, and quality assessment may not be always according to the highest standards. Studies from these regions or countries were not available in the former edition of the vitamin D book. For the fifth edition of the vitamin D book, we made an attempt to cover most regions and countries of the world.

#### 3.1 Vitamin D status in North America (including Canada and Mexico)

Several large studies examined the vitamin D status in North America. Data of a selection of studies are shown in Table 54.1. The table includes data on country, latitude, study population, age, serum 25(OH)D assay method, mean levels of serum 25(OH)D in nmol/L, and percentage of the population with serum 25(OH)D below 25 nmol/L and below 50 nmol/L if available.

In North America (including Canada and Mexico), the latitudes ranged from 76°N to 19°N. The largest study is the National Health and Nutrition Examination

Survey (NHANES). This US study comprised a large, nationally representative sample and presents the vitamin D status of men and women, in different age groups, and according to different ethnic backgrounds [25,26]. The most recent of these two studies reported the vitamin D status of 16,180 persons aged 1 year or older in the period 2011–14 [25]. The prevalence of vitamin D deficiency (<30 nmol/L) was slightly lower in men (4.4%) than in women (5.7%). Furthermore, the prevalence was lowest in 1 to 5-year-olds (0.5%), peaked in adults aged 20–39 years (7.6%), and was lower again in adults aged 60 years and older (2.9%) [25]. Finally, the prevalence was highest in non-Hispanic black (17.5%) and lowest in non-Hispanic white (2.1%) [25]. In the Canadian Health Measure Survey (n = 11,009), males had lower 25(OH)D levels than females, and immigrants had lower serum 25(OH)D than nonimmigrants. Furthermore, the lowest 25(OH)D levels were found in the age groups 12–17 years (55.8 nmol/L) and 18–64 years (58.5 nmol/L) as compared with the age groups <5, 5–11, >64 years [17]. A relatively high prevalence of vitamin D deficiency (<25 nmol/L) was observed in refugee women in Canada (21%) [21] and in pregnant women in the United States (22.3% < 30 nmol/L) [28]. In addition, high prevalence rates of low vitamin D status (<37.5 nmol/L) were found in neonates (23%–31%) and in cord blood (38.9%) [32,35,40]. In a review of studies in Aboriginal groups in the Canadian Arctic, the prevalence of low vitamin D status ranged from 13.9% to 76.0% among children and adults in the summer [44].

#### 3.2 Vitamin D status in South America

During the past decade, many studies have been published on vitamin D status in South American countries (Table 54.2). Latitudes range from 4°N to 33°S. By far, most studies were published in Brazil. In a metaanalysis of 72 Brazilian studies, the mean serum 25(OH)D status was 67.7 nmol/L in the period 2000–17 and 28.2% had a serum 25(OH)D level below 50 nmol/L [84]. In several studies, a very high prevalence of persons having serum 25(OH)D below 50 nmol/L was observed, especially in Colombian toddlers (42.5%) [45], Brazilian children aged 11–15 months (68.2%) [49], community-dwelling elderly in Sao Paulo, Brazil (58.0%) [76], female Xavante Indians (43.4%) [73], Koya Indian children living in Argentina (96.6%) [80], healthy adults aged >60 years from Chile (48.0%) [82], and adult and older women from Chile (51.6% and 64.9%, respectively) [83]. In another study, performed in a random sample of elderly in Brazil, 40.8% had a serum 25(OH)D < 75 nmol/L [53]. Finally, 71.2% of nursing home residents living in Natal, Brazil, had a serum 25(OH)D < 30 nmol/L [51].



**TABLE 54.1** Vitamin D status and prevalence of vitamin D deficiency in North America (including Canada and Mexico).

References	Country Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D			Comments
						Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
Canada									
Yousef [17]	Representative sample (CHMS 2012/13 & 2014/15)	Men and women	11,009	39.2 ± 0.1 (3–79)	CLIA	Total: 60.3 Nonimmigrant: 62.7 Immigrants: 51.2	Total: 10.3 (<30) Nonimmigrant: 7.8 Immigrant: 19.0	Total: 63.6 Nonimmigrant: 31.8 Immigrant: 52.8	
Munasinghe [18]	Representative sample (CHMS 2012/13)	Children	2270	3–18	CLIA	62.2 (55.8–68.7) <sup>a</sup>	5.6 (<30)	29.1	
Skogli [19]	Northern Canada (36 communities), 54–76°N	Inuit	1649	42 ± 15.1	CLIA	Total: 58.5 ± 33.9		45.7	DEQAS
Chao [20]	Majority from Northern Alberta 53 ± 3°N	Workers	6101	42 ± 14	NM	84 ± 42	3 (<27.5) 8 (<37.5)	40 (37.5–75)	
Aucoin [21]	Calgary 51°N	Refugee:			RIA/CLIA				
		Women	461	20–45		46.2 (44.1–48.3) <sup>a</sup>	21	61	
		Children	756	0–19		55.5 (53.8–57.2)	10	42	
Lacroix [22]	Sherbrooke 45°N	Pregnant women (6–13 weeks)	655	28.4 ± 4.5	LCMS	63.0 ± 18.8		26.7	
El [23]	Greater Montreal (CHMS) 45°N	Preschoolers	508	2–5	CLIA	74.4 (60.3–93.5) <sup>b</sup>		4.5 (<40)	
Omand [24]	Toronto 43°N	Healthy children from practice-based network:		Median 36 months (1–6 years)	CLIA	Median total: 83		Increased odds on <50 nmol/L in non-Western: OR = 1.9 (1.3–2.9)	DEQAS
		Non-Western	421			85			
		Western	1119			89			
United States									
Herrick [25]	Nationally representative sample (NHANES)	NHANES 2011–14	16,180	1+ - 60+ (6 different age groups)	LCMS		5 (<30)	23.3	
Liu [26]			26,010				6.4 (<30)	28.9	NIST

	Nationally representative sample (NHANES)	NHANES 2001–10		45.6 (45.1–46.2) <sup>a</sup> (18+)	LCMS (2001–06 RIA, adjusted + converted to LCMS)				
Rapson [27]	Minneapolis, MN; Forsyth Co., NC; Washington Co., MD; Jackson, MS.	Prospective cohort of adults aged 45–64 years free of peripheral arterial disease (ARIC)	11,789	56.8 ± 5.7	LCMS	Total: 60.7 ± 21.2 White: 65.2 ± 20.7 Black: 47.2 ± 16.7		White: 22 Black: 61	
Gernand [28]	12 medical centers	Pregnant women (≤26 wks)	2048	<20–30+	LCMS	51.2 ± 27.2	22.3 (<30)	55.4	
Shea [29]	Random sample of well-functioning older adults (70–81 years) (Health ABC study)	Older adults: black white	977 1607	70–81	RIA	52.5 ± 26.0 73.0 ± 27.3	9 2	54 18	
Fohner [30]	Rural Southwest Alaska, 63°N	Yup'ik Alaska native people	743	14–93	LCMS	77.6 ± 2.5		22.9	NIST
Young [31]	Rochester 43°N Baltimore, 39°N	Pregnant adolescents (≈26 weeks) Cord blood	168	17.1 ± 1.1	RIA	55.3 ± 25.5 52.0 ± 25.5		50	DEQAS
Kanike [32]	Cleveland, 41–42°N	Retrospective chart review, neonates	1517		LCMS	Median: 47.4	31 (<37.5)	80 (<75)	
Akinlawon [33]	Massachusetts, 41–42°N	Puerto Rican adults	822	45–75	RIA		13 (<30)	56	
Penrose [34]	Massachusetts, 41–42°N	Refugees	2610	23 median age at arrival	NM			43	25(OH)D < 50 nmol/L most prevalent in refugees from the Middle East
Marshall [35]	New Jersey, 40°N	Cord blood	265		LCMS		38.9 (<37.5)	68.7	
Mirfakhraee [36]	Dallas county 32°N	Multiethnic population sample, African Americans oversampled, longitudinal:	2045		EIA				Samples analyzed in same batch, significant longitudinal changes

Continued

**TABLE 54.1** Vitamin D status and prevalence of vitamin D deficiency in North America (including Canada and Mexico).—cont'd

References	Country Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D			Comments
						Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
			2000–02	44		42.7		60.6	
			2007–09	51		39.4		66.4	
<b>Mexico</b>									
Carrillo-Vega [37]	Representative sample	Community-dwelling older adults	1088	69.6 ± 7.7 (all 60+)	CMIA	57.7 ± 20.2		Total: 36.9 Men: 26.3 Women: 46.8	
Contreras-Manzano [38]	Nationally representative sample	Women	3260	20–49	CMIA			37.2	Quality control: NIST 968E
Flores [39]	Nationally representative sample	Children	366 659	2–5 6–12	ELISA	78.3 105.8	0.5 (<20) 0.2 (<20)	24.6 10.2	
Moodley [40]	Tijuana 32°N	Mother Newborns	49 49	18+	LCMS	65.5 47.3	10 (<37.5) 23 (<37.5)		Baseline data RCT (preterm, low birth weight excluded)
Denova-Gutiérrez, [41]	Mexico city 19°N	Representative sample of school children	533	11.6 ± 3.9 (5–20)	CLIA	54.2 ± 16.2		Total: 42.9 Boys: 35.9 Girls: 51.2	
López-Bautista [42]	Mexico city 19°N	Volunteers open population without clinical signs of coronary heart disease	1467	53.3 ± 9.3	CLIA			Total: 38.5 Men: 28 Women: 48.9	
Clark [43]	Toluca 19°N	Healthy subjects	585	41.1 ± 15	LCMS	52.3	2.0	43.6	

<sup>a</sup>(95% confidence interval).<sup>b</sup>interquartile range.

AMK, automated kit; CLIA, chemiluminescence immunoassay; CMIA, chemiluminescent microparticle immune assay; ECLIA, electrochemiluminescence immunoassay; EIA, enzyme immunoassay; ELISA, enzyme-linked immune sorbent assay; HPLC, high-performance liquid chromatography; LCMS, liquid chromatography tandem mass spectroscopy; NM, not mentioned; RAIA, random access immunoassay; RIA, radio immune assay.

**TABLE 54.2** Vitamin D status and prevalence of vitamin D deficiency in South America.

References	City, state Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D		Comments
						Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %
Beer [45]	Columbia 4°N	National nutrition survey:			NM			
		Toddlers	2104	1-<2		59.7 (SE: 0.9)	3.1 (<30)	42.5
		Preschoolers	6813	2-<5		65.2 (SE: 0.6)	1.9	27.7
		Schoolchildren	16,454	5-<13		65.9 (SE: 0.5)	2.4	21.8
		Adolescents	6470	5-<18		67.3 (SE: 0.6)	3.0	20.4
		Pregnant women	1262	11–49		59.5 (SE: 1.1)	6.7	33.3
Orces [46]	Andes mountains and coastal regions, Ecuador	Nonpregnant women	7170	18–49		64.3 (SE: 0.6)	3.7	23.8
		Older adults participating in national health survey	2374	71.0 ± 8.3	LCMS	60–69 years 69.0 ± 28.8 70–79 years 63.8 ± 24.3 ≥80 years 64.8 ± 33.0		21.6
Lima-Costa [47]	Brazil	Nationally representative sample	2264	62.6 (61.7–63.6) <sup>c</sup> (50+)	CMIA	66.8	1.7 (<30)	16
De Oliveira [48]	Four Brazilian cities	School-based study in adolescents	1152	12–17	CMIA	70.9 (68.4–72.9) <sup>c</sup>		21
Lourenço [49]	Four Brazilian cities	Children 11–15 m old	468	13.4 ± 1 months	HPLC	38.1 (26.2–55.7) <sup>b</sup>	32.9 (<30)	68.2
Monteiro Junior [50]	Alcântara, Brazil 2S	Afrodescendant individuals, inhabitants of Quilombola communities	382	57.8 ± 15.3	ECLIA	125.8 ± 33.7		0.81
Sousa [51]	Natal, Brazil, 5S	Nursing home residents without marked physical and mental impairment	153	81.7 ± 9.2 (60–103)	CLIA	59.7 (56.9–65.2) <sup>c</sup>		71.2 (<30)
Ceolin [52]	Southern Brazil	Population-based study of older noninstitutionalized elderly	577	60+	CMIA	M: 71.6 (68.1–75.4) <sup>c</sup> F: 63.4 (61.2–65.4)		25.5
Issa [53]	Joao Pessoa, Brazil 7 S	Random sample of elderly	142	60+	HPLC	64.1 ± 8.2		40.8 (<75)
Cobayashi [54]	Acrelandia, Brazil 9°S	Amazonian children	974	5.4 ± 2.8	HPLC	66 <sup>b</sup> [18,19,55–72]		11.0
Abrahão [73]	Two indigenous reserves (Sangradouro and Sao Marcos), inMato Grosso state, Brazil, 15°S	Xavante Indians	819	41.8 ± 19.0 (18+)	ECLIA	Spring/summer: 72.6 ± 31.5 Autumn/winter: 65.4 ± 25.5		27.7 M: 12 F: 43.4
Milagres [74]	Viçosa, Minas Gerais, Brazil, 21°S	School children	378	8–9	CLIA		0	12.2

Continued

TABLE 54.2 Vitamin D status and prevalence of vitamin D deficiency in South America.—cont'd

References	City, state Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D		Comments
						Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %
Benaïm [75]	Rio de Janeiro, Brazil 23°S	Healthy pregnant women:	180	26.8 ± 5.6	LCMS			
		- Gestational week 5–13				65.6 ± 17.5	1 (<30)	15
		- Week 20–26				78.6 ± 21.8	0	11
		- Week 30–36				84.0 ± 24.6	1	10
Lopes [76]	Sao Paulo, Brazil 23°S	Community-dwelling elderly	908	72.8 ± 4.8	RIA	48.5 ± 23.3	14.4	58.0
Martini [77]	Sao Paulo, Brazil 23°S	Population-based sample	636		HPLC	Boys 28.1 ± 10.6 Adult men 48.4 ± 22.9 Elderly men 50.9 ± 21.9 Girls 33.2 ± 14.7 Adult women 51.0 ± 26.1 Elderly women 53.9 ± 18.9		Differences in season of sampling between life stages
Urrunaga-Pastor [78]	Peru	Euthyroid nondiabetic patients outpatient service private clinic	204	38.5 ± 10.6 (18–59)	NM	62.4 (47.4–82.4) <sup>b</sup>		29.4
Tomaino [79]	Lima 12°S Tumbes 3°N S, Peru	Population-based sample of adolescents	1074	14.9 ± 0.8	CLIA	Lima: 52.0 ± 14.3 Tumbes: 75.3 ± 23.0		
Hischler [80]	San Antonio de los Cobres, Argentina 24°S	Koya Indian children from 4 schools	290	10.6 ± 2.9 (4–19 years)	RIA	25.1 (19.9–52.1) <sup>a,b</sup>	49.7	96.6
Terán [81]	Bolivia	School children		6–12	ELISA			
	- Taraco 16°S	- Rural	120			66.0 (SEM 2.7)	11 (<30)	
	- Caranavi 15°S	- Semiurban	96			79.4 (SEM 4.8)	3	
Vallejo [82]	Santiago, Chile 33°S	Healthy adults	1329	51.6 ± 17.0 (18–89)	ECLIA	55.2 ± 23.7		<40 years: 36.5 >60: 48.0
Solis-Urra [83]	Representative sample of Chile	Noninstitutionalized			LCMS			51.6 64.9
		- Adult women	1245	35.4 ± 8.5		50.4 ± 20.0	16.4 (<30)	
		- Older women	686	73.6 ± 6.6		44.9 ± 21.2	26.4 (<30)	

<sup>a</sup>Because of overlap with Hirschler [80], only the results of Buenos Aires boys were reported.<sup>b</sup>Median (interquartile range).<sup>c</sup>(95% confidence interval).



### 3.3 Vitamin D status in Europe

Vitamin D status has been studied extensively in many European countries in different age groups (Table 54.3). Recently, a review on current vitamin D status in European and Middle East countries and strategies to prevent vitamin D deficiency has been published [16]. Last years, more studies from Eastern Europe have been published, as well as a review paper [111]. Data from Iceland, Norway, Ireland, the United Kingdom, the Netherlands, Germany, and Greece have recently been standardized by the European ODIN study [11], making all these data comparable. In the ODIN Study serum 25(OH)D was lower than 30 nmol/L in 12.5% of the participants and lower than 50 nmol/L in 40%. A general trend in these data was that vitamin D status was usually better in Nordic countries than around the Mediterranean despite higher latitude. This difference may be caused by the traditional high intake of cod, cod liver, and cod liver oil in Norway and Sweden [112]. In Finland, fortification of dairy products has been increased in two steps, leading to a formidable improvement of vitamin D status [88]. Representative data have been obtained in the United Kingdom in the National Dietary and Nutrition Survey [113] (Fig. 54.2). As expected, serum 25(OH)D was lower in older persons than in adults. Unexpectedly, serum 25(OH)D was low in adolescents [11,91]. Vitamin D status was poor in older persons and patients with hip fracture and the institutionalized. Similarly, very low serum 25(OH)D levels were observed in noninstitutionalized elderly in Switzerland. Also, in Italy and Greece, very low serum 25(OH)D levels were observed, while sunshine is abundant in these countries. This may be caused by a more pigmented skin and by sun avoidance behavior especially in summer because of the high temperatures. Vitamin D status usually is very poor in immigrants from non-Western countries [114,115], compared with native people (Fig. 54.3). This is even worse in pregnant non-Western immigrants, in which mean serum 25(OH)D was lower than 25 nmol/L in midwives practices in the Hague [116].

### 3.4 Vitamin D status in Middle East

Serum 25(OH)D is lower in these countries than should be expected based on the abundance of sunshine (Table 54.4). A recent review on vitamin D status in the Middle East showed widespread vitamin D deficiency [134]. In Turkey, Saudi Arabia and Kuwait serum 25(OH)D was lower in women than in men [119,127,130]. In women, vitamin D status depended on clothing style being lower in traditionally clothed

women than in women with Western style clothing. A very low serum 25(OH)D was observed in Saudi Arabia even with the very sunny climate. This may be explained by the often completely covered skin in this country. Similar trends were visible in Egypt and Iran. Another possible explanation for the low levels of 25(OH)D in this part of the world is the extreme heat, leading people to avoid being outdoors during the sunny parts of the day.

### 3.5 Vitamin D status in Asia

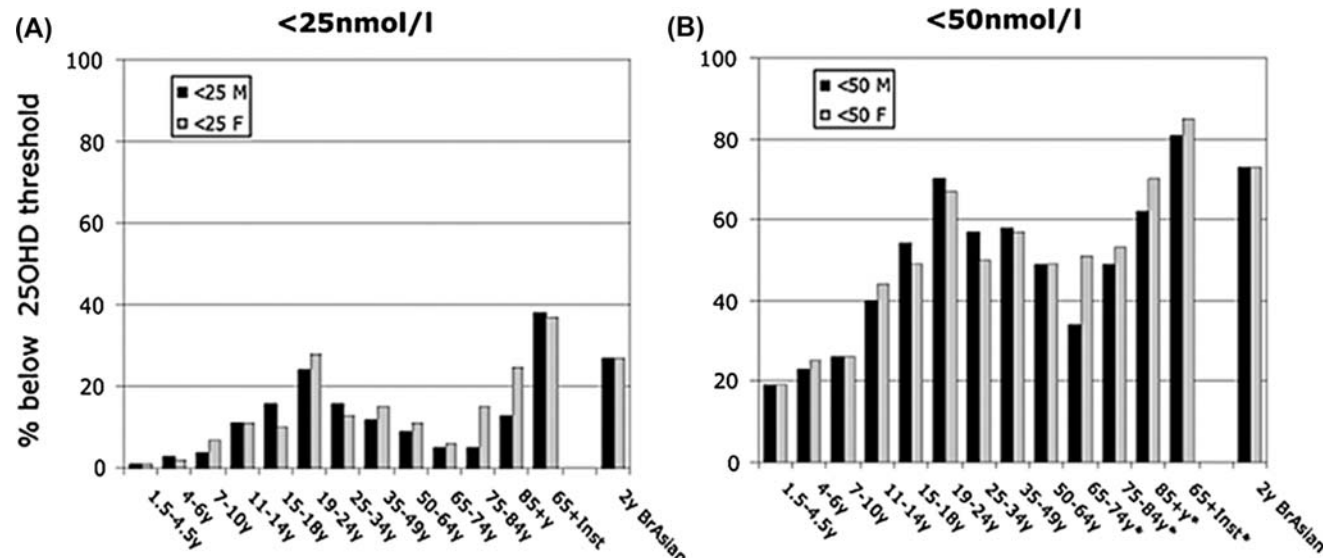
Recently, many studies on vitamin D status in Asian countries have been published although there remain Asian countries with a complete lack of published data such as Myanmar and Uzbekistan (Table 54.5). A very low vitamin D status was observed in Mongolia with a prevalence of serum 25(OH)D below 25 nmol/L in 88.6% of the young women and around 40% of children [135–137]. Clinical signs of rickets are still very common in Mongolia [113,136]. Most studies on vitamin D status in Asia have been performed in China. A nationwide population-based study demonstrated 25(OH)D levels below 50 nmol/L in 80% of the children above 6 years old [143]. Also, very high prevalence of 25(OH)D concentration below 50 nmol/L is reported in lactating women in China (97.9%) [148]. Large differences in vitamin D status in China are observed dependent on latitude and altitude [138,141,142,144]. For example, a very high prevalence of 25(OH)D concentrations below 50 nmol/L of 82.2% was observed in women living in a sunlight-deprived basin in Sichuan and a very low prevalence of 2.9% in adults living in Hainan, a tropical island province in southern China [138,141]. A recent review demonstrated that within the South Asian countries (Bangladesh, India, Pakistan, Nepal, Bhutan, Maldives, Sri Lanka, and Afghanistan), 25(OH)D concentrations below 50 nmol/L are more common than would be expected from the latitude.

The highest prevalence of 25-hydroxyvitamin D concentrations below 50 nmol/L within the South Asian countries was found in Pakistan (73%) followed by Bangladesh (67%), India (67%), Nepal (57%), and Sri Lanka (48%) [176]. This may be due to pigmented skin, skin covering, and sun avoidance behavior. Also, air pollution probably plays a role as 25(OH)D concentrations are particularly low in large cities such as Delhi with mean concentration of 20–25 nmol/L in adults [168,170]. Among Southeast Asian countries such as Singapore, Thailand, Cambodia, Taiwan, and Malaysia, vitamin D status is much better probably mainly because these Asian countries are localized closest to the equator.

**TABLE 54.3** Vitamin D status and prevalence of vitamin D deficiency in different European countries.

References	Country Latitude°N/S	Study population	N	Age Years	25(OH)D				Comments
					25 (OH)D method	Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
Cashman [11]	Iceland 64°N Regionally representative	Adult men and women	5519	66–96	LCMS	57.0 ± 17.8	4.2	33.6	ODIN
Cashman [11]	Norway (Tromso) latitude 69°N	Regionally representative	12,817	30–87		65.0 ± 17.6	0.3	18.6	ODIN
Cashman [10]	Norway (Oslo) 60°N		866	30–76		71.0 ± 19.5 (white)	0.1 (white)	14.9 (white)	
Petrenya [85]	Norway 68 = 70°N	Population-based	4465	55.9 ± 8.5	CLIA	64.0 ± 19.2		25	
Buchebner [86]	Sweden 56°N	OPRA women	995	80 (80–81)	LCMS	78 ± 30	0	16	
Nälsen [87]	Sweden	Nationally representative	206 children 268 adults	10–12 18–80	LCMS LCMS, CLIA	52.9 ± 14.3 63.5 ± 18.2	4.9 (<30) 3 (30)	42.2 39	
Cashman [10]	Finland 60–70°N	Nationally representative	4102	29–77		67.7 ± 13.2	0.2	6.6	
Jaaskelainen [88]	Finland 60–70°N	Nationally representative	H2000: 6134 H2011: 4051	≥30 years mean 56 years	RIA CLIA LCMS-VDSP	47.6 65.4	13.0 0.6	55.7 9.1	
Ikonen [89]	Finland 66–70°N	Northern Finland representative	3650 samples in 1997, and 2013	1997: 31 2013: 46	LCMS and CMIA	54.2 ± 18.5 64.8 ± 19.4	9.5 (<30) 2.5 (<30)	42.7 23.5	
Cashman [10]	Denmark (Copenhagen) 56°	Regionally representative	3409	19–72		65.0 ± 19.2	0	23.6	
Cashman [11]	UK 50–59°N	Children, teens and adults Nationally representative	1488	1.5–91		47.4 ± 19.8	15.4	56.4	ODIN
Aspell [90]	UK 50–55°	Nationally representative	6004	>50–80+	Diasorin Liaison	48.7 ± 23.4	26.4 (<30)	57.3	
Carson [91]	Northern Ireland 54–55°N	Girls and boys Regionally representative	1015	11 and 15 years	ELISA	41.9 ± 16.0	16.7	66.2	
Cashman [92]	Ireland 51–54°N	Nationally representative	1118	18–84	ELISA-VDSP	56.4 ± 22.2	6.0	45.0	
Ni Chaoimh [93]	Ireland 51°N	Cork birth cohort	741	2	LCMS	63.4 ± 20.4	1.6	26.7	
Laird [94]	Ireland 50–55°N	Nationally representative	5356	50–98	LCMS	51.3	13.1	53.7	TILDA
Cashman [11]	Netherlands 52°N	LASA 2009 Nationally representative	915	61–99		64.7 ± 22.6	2.4	28.5	ODIN
Cashman [11]	Netherlands 52°N	Regionally representative	2625	40–66		59.5 ± 21.7	4.9	33.6	ODIN
Hoge et al. [95]	Belgium 51°N	Adults	697	42.7 (32–53)	CLIA	49.3 (35–65)	7.3	51.1	
Courbebaisse [96]	France 47–49°N	Pregnant women Neonates	2327 1763	31.5 ± 5.0 Cord blood	Diasorin RIA	79.5 ± 28.8 42.5 ± 18.0	1.2 13.1	14.0 68.5	

Cashman [11]	Germany 47–55°N	Nationally representative	6995	18–79		50.1 ± 18.1	4.2	54.5	ODIN
Cashman [11]	Germany 48–52°N	Nationally representative Children and adolescents	10,015	1–17		54.0 ± 19.2	6.0	44.5	ODIN
Gellert [97]	Germany	Pregnant women Nonpregnant women	429 429	29.9 ± 4.8 29.8 ± 5.1	Diasorin CLIA	35.5 ± 20.0 50.0 ± 22.8	36 12	78.1 53.9	
Souberbielle [98]	France 43–49°N	Variété study	892	18–89	CLIA	60 ± 20	6.3	34.6	
Rabufetti [99]	Switzerland	Military training	1045	18–19	CLIA	68 (55–82)	2.3	17	
Santos [100]	Portugal (incl Madeira, Azores)	Nationally representative	1500	65–100	ECLIA	42.3 ± 29.7	39.6 (<30)	70	
Duarte [101]	Portugal	Nationally representative	3092	18–>75	Comp immunoassay	42 ± 17	21.2	66.6	
Bleizgys [102]	Lithuania	One lab	9581	33 ± 23	immunoassay	70.8 ± 9.4		67.3 (<75)	
Karonova 59–61°N [103]	Russia 59–61°N	Outpatients	1544 adults 120 children	18–75 7–17	ECLIA	54.8 46.8	8 (<30)	45.7	
Wyskida [104]	Poland	Random selection	3472	83.2 ± 8.7	ELISA	51.2 ± 24.0	F 12.7 M 7.9		
Chlebna-Sokol [105]	Poland 50–53°	Regionally representative	720	9–13	ECLIA		March 20.2 October 0.1	March 84.2 October 26.0	
Sochorova [106]	Czech Republik	Children clinics	419	5 or 9	ECLIA	63 (median)	3	27	
Sokolovic [107]	Bosnia Herzegovina	Sarajevo university	2483	All ages	Elecsys	48 ± 31	25.9	60.6	
Karin [108]	Croatia	Mandatory preschool examination	260	5–6	ECLIA	46.6 ± 20,2	12	58	
Manios [109]	Greece	Regionally representative	2386	9–13	Elecsys	50.1 ± 13.0	5.2 (<30)	52.5	
Dimakopoulos [110]	Greece	Population study	1084	36 (median)	ECLIA	42 (median)	28.8 (<30)	65.2	
Cashman [11]	Greece 35–40°N	Regionally representative	806	9–14		47.3 ± 12.5	2.2	62.4	ODIN
Cashman [11]	Greece 37°N	Regionally representative	222	3–6		54.3 ± 15.7	1.4	40.5	ODIN



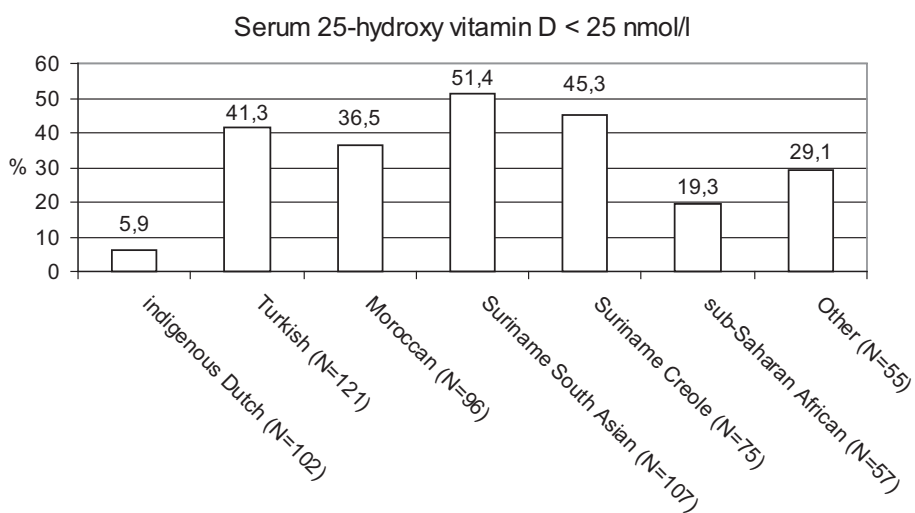
**FIGURE 54.2** Percentage of the population in the United Kingdom with serum 25-hydroxyvitamin D lower than 25 nmol/L (vitamin D deficiency) or lower than 50 nmol/L (vitamin D deficiency and low vitamin D status). Data from the National Dietary and Nutrition Survey [113]. The prevalence of low serum 25(OH)D levels is remarkably high in adolescents and young adults.

### 3.6 Vitamin D status in Africa

Africa is the continent with most sun hours per year and the largest exposure to UV-B radiation particularly in the countries situated around the equator. Therefore, African populations have long been considered to have a low prevalence of vitamin D deficiency. Reliable, population-based data on vitamin D status in different African areas remain scarce as compared with other continents, but in the past 10 years, a considerable amount of studies have been published. The literature was recently reviewed in a meta-analysis [177]. A selection of studies from Africa is summarized in Table 54.6. In the meta-analysis, the estimated prevalence of 25(OH)D concentrations below 30 nmol/L is approximately

one in five people living in Africa, underlining that even in the sunniest continent, deficient vitamin D status is common. However, large variation in vitamin D status is present. Vitamin D deficiency is particularly common in populations living in northern African countries or South Africa compared with sub-Saharan Africa. For example, the mean 25(OH)D concentration from studies in Morocco is 44.5 nmol/L, whereas in countries around the equator such as Kenya or Uganda, mean concentrations are around 70–80 nmol/L [177–179,191,195]. In South Africa, 25(OH)D concentrations below 50 nmol/L are particularly common in specific populations such as Africans from Asian Indian origin, newborns, and pregnant women [177,197,198,201].

**FIGURE 54.3** Prevalence of vitamin D deficiency (serum 25-hydroxyvitamin D < 25 nmol/L) in different ethnicities in the Netherlands. Data from Van der Meer et al. [115].



**TABLE 54.4** Vitamin D status and prevalence of vitamin D deficiency in Middle East countries according to different studies.

References	Country Latitude°N/S	Study population	N	Age Years	25(OH)D				Comments
					25(OH)D method	Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
Buyukuslu [117]	Turkey	Female students	100	20.9 ± 2.1	ELISA	65.7 ± 25		34.0 Covered 55% Uncovered 20%	Istanbul; low levels related to clothing style
Hocaoglu-Emre [118]	Turkey	School children	640	6–9	CLIA	65 (median)	3.8 (<30)	16.9	
Göktas [119]	Turkey	Family health centers	11,734	46.5 ± 16.9	ECLIA	41.5 ± 28.8		Women 74.4 Men 57.2	
Yesiltepe-Mutlu [120]	Turkey		108,742	0–>70	LCMS	54 ± 33 <1 year: 93 (mean) 1–10 years: 67.8 11–18 years: 48.0	27 7 8 26	51 13 30 58	Successful supplementation in 0–1 year
Azizi [121]	Afghanistan	Adolescents	308		CLIA		45	79	
Hosseinpanah [122]	Iran	Healthy adults	251	56.7 ± 11.7	EIA	45.2 (27.5–77.5)	19.1	53.8 (<37.5)	Controls of a cardiovascular outcomes study
Nikooyeh 29–37° [123]	Iran	Children	667	5–18		27.3 ± 17.6			
Saliba [124]	Israel	Men Women	198,834	0–>80	CLIA	54.8 ± 24.2 50.7 ± 24.6	10.0 16.2	45.0 51.8	
Merzon [125]	Israel		7025	0–103 mean 47.4			13.1		
Nichols [126]	Jordan 31°N	Women	2032	15–50	LCMS	27.5 (22.7–33.7)	60.3 (<30)	95.7	Prevalence vitamin D deficiency: Hijab 1.6×; niqab 1.9×
Hussain [127]	Saudi Arabia	Men Women	3363 7346	0–>60	HPLC	50.5 41.9	23.7 35.6	63.9 (m + w)	Serum 25(OH) D < 25 nmol/L in 49% of adolescents; vit D deficiency in Saudi 37.2%; in non-Saudi 20.3%
Alfawaz [128]	Saudi Arabia	Men Women	756 2719	46.9 ± 16.3	HPLC	35.5 ± 30.6	36.1 48.8	72.4 78.1	

Continued



**TABLE 54.4** Vitamin D status and prevalence of vitamin D deficiency in Middle East countries according to different studies.—cont'd

References	Country Latitude°N/S	Study population	N	Age Years	25(OH)D				Comments
					25(OH)D method	Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
Al-Daghri [129]	Saudi Arabia	Multiple cohorts	7360	28.2 ± 15.3	ECLIA	40.3 ± 24.1	29.4	73.2	Improving trend in vitamin D status
Al-Taïar [130]	Kuwait 29,4°	Adolescents	Boys 694 Girls 722	11–16	LCMS	39.8 (median) 21.5 (median)	39.5 (overall)	70.3 91.7	
Narchi [131]	United Arab Emirates	Female adolescents	293	15.3 ± 2.0	ECLIA	21.5 ± 10.0	78.8	98.6	Low levels related to clothing style
Zainel [132]	Qatar	Primary healthcare centers	102,342	18–65			14.1	71.4	
Olama [133]	Egypt	Healthy women	50	33.1 ± 9.7	ELISA	47.0 ± 13.5	6 (<20)	30	Controls of a fibromyalgia study

**TABLE 54.5** Vitamin D status and prevalence of vitamin D deficiency in Asian countries according to different studies.

References	Country Latitude°N/ S	Study population	N	Age Years	25(OH)D method	25(OH)D			Comments
						Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
Bater [135]	Mongolia 48°N	Children	9595	9.4 ± 1.6 (6–13)	ELFA		40.6		Ulaanbaatar. DEQAS.
Uush [136]	Mongolia	Children Young women	524 867	6–59 months	ELISA		42.2 (<23) 52.2 (<23)		4 economic regions of the country and Ulaanbaatar
Ganmaa [137]	Mongolia 48°N	Women (mothers)	420	34.9 ± 4.8	LCMS	19.0 ± 10.0	88.6 (<30)	98.8	Ulaanbaatar
Lin [138]	China 18–20°N	Men and women, population-based	1690	48.3 ± 13.5	ECLIA	94.2 ± 26.9	0	2.9	Hainan province
Lin [139]	China 34.2–35.5°N	Healthy women, population-based	1151	18–40	HPLC	52.4 median (IQR 34.0 –86.5)	16.0	47.8	Henan province
Yu [140]	China 30.4°N	Men Women With healthcare checkup	25,691 22,146	42.7 ± 12.9	CLIA	56.9 ± 19.7 46.0 ± 17.2			Wuhan
Fan [141]	China 26 –34°N	Healthy women Population-based Basin Plateau	1057 337	58.2 ± 13.9 59.6 ± 14.9	ELISA	40.7 ± 15.6 52.5 ± 19.9	21.9 (<30) 101 (<30)	82.2 50.5	Sichuan province
Yang [142]	China	Children 0–18 years admitted to 825 hospitals	460,537	1.3 median	LCMS	72.2 ± 30.1	6.7 (<30)	22.6	18 provinces in mainland China
Li [143]	China	Children 6–18 years Population-based	10,696	12.4 ± 3.7	CLIA	39.3	30 (<30)	80	Nationwide
Liu [144]	China 38°N	Women and men Population-based	11,157	53.6 ± 10.7	CLIA	34.5 median (IQR 27.5 –44.5)	17.3	81.9	Gansu province
Fang [145]	China 39°N	Women and men	1814	>18 years	CLIA	49.4 ± 14.9	7.7 (<30)	52.3	Tianjin
Cheng [146]	China 31°N	Ambulant men and women >65 years	1677 men 2247 women	72	ECLIA	60.3 52.5	2.7 5.9	35.5 48.9	Shanghai
Han [147]	China 27 –31°N	Men and women, population-based	6597	52.5 ± 13.5	CIA	41.1 ± 10.8		83.3	Shanghai, Jiangxi province, Zhejiang Province
Zhao [148]	China 23 –48°N	Lactating women	2004	27.1	RIA	15.8 median (IQR 10.5 –24.0)	85.3 (<30)	97.9	Beijing and Shanghai municipalities, Guangdong, Heilongjiang, Yunnan, Gansu, Zhejiang and Shandong provinces
Cheng [149]	China	Healthy men Healthy women	2948 3066	67.5 median (IQR 62.3 –73.2)	RIA	61.0 median (IQR 44.3 –80.6)	7.8 12.0	34.1 44.0	CNNHS, nationally representative sample,

TABLE 54.5 Vitamin D status and prevalence of vitamin D deficiency in Asian countries according to different studies.—cont'd

References	Country Latitude°N/ S	Study population	N	Age Years	25(OH)D method	25(OH)D			Comments
						Mean ± SD nmol/L	<25 nmol/L %	<50 nmol/L %	
Ning [150]	China 39.9°N	>60 years, population- based Men and women, health checkup	5531	66.8 median (IQR 63.0 –72.5)	ECLIA	53.7 median (IQR 38.8 –71.0) 30.8 ± 18.8	44.7	87.1	RIA validated against LCMS Beijing, age stratified in groups
Xiao [151]	China 31.5°N	Pregnant women	5823	26.4 ± 3.1	ELISA	34.0 median	40.7 (<30)	78.7	Wuxi
Zhen [152]	China 36°N	Women 7136 Men 2902	7136 2902	40–75	EIA	39.2 ± 17.8 45.3 ± 15.7		75.2	Lanzhou
Xu [153]	China 22°N	Children Adults Adults Adults	1165 933 544 51	6–17 18–44 45–64 65+	ELISA	39–53 * 42–57 47–69 41–56			Hong Kong
Yang [154]	Japan	Children birth cohort study	4655	2	LCMS	61.8 (median)		24.7	15 regional health centers validated through Accuracy-Based Vitamin D Survey
Nakamura [155]	Japan 38°N	Men Women Population-based	9084	60.1 ± 9.3 59.3 ± 9.2	CLIA	55.9 ± 18.8 45.2 ± 16.6		53.6	Niigata prefecture
Park [65]	Korea	Men Women	1145 1204	>10 years	RIA	43.2 39.2		75.2 82.5	6th Korea NHANES Representative national database Age only in categories
Choi [156]	Korea	Women Men	3878 3047	45.0 ± 19.3 42.4 ± 19.6	RIA	45.5 ± 17.7 53.0 ± 18.7	10.4 4.7	64.5 47.3	4th Korea NHANES
Kim [157]	Korea	Adolescents boys Adolescent girls	1095 967	10–18 10–18	RIA	45.9 ± 15.7 42.4 ± 14.7	11.7 15.4	64.2 72.6	4th Korea NHANES
Ahmed [158]	Bangladesh	Pregnant women (gestational age <20 weeks)	155	23.6 ± 4.8	ECLIA		17.3 (<30)	64.5	Three geographical regions
Lee [159]	Taiwan 25°N	Adults Healthy with normal kidney function	3954	55.5 ± 12.6	ECLIA	72.3 ± 25.8		22.4	Four districts in northern Taiwan
Chee [160]	Malaysia 3°N	Boys Girls	127 116	10.2 ± 0.9 10.0 ± 1.0 (9 –11)	LCMS	50.3 ± 13.7 36.8 ± 11.9	3.9 (<30) 35.3 (<30)	52.8 87.9	Kuala Lumpur

Moy [161]	Malaysia 3°N	Participants of intervention study Adults men women	158 222	48.5 ± 5.2	CLIA	56.2 ± 18.9 36.2 ± 13.4		67.9	Kuala Lumpur
Laillou [162]	Vietnam	Women Children	541 485	32.9 3.7	HPLC	44.5 43.4	17 (<30) 21 (<30)	57 58	Nationwide sample
Pratumvinit [163]	Thailand 13.5°N	Pregnant women	147	28.9 ± 6.4	ECLIA	61.6 ± 19.3	0.7	34	Bangkok
Smith [164]	Cambodia	Women	725	15–49	ELISA	69.7 ± 31.2	4.1	29	Nationally representative sample
Loy [165]	Singapore 1.3°N	Pregnant women	940	30.5 ± 5.1	LCMS	81.0 ± 27.2			41% < 75 nmol/L
Bi [166]	Singapore 1.3°N	Men Women	59 55	30.9 ± 11.9 32.2 ± 13.0	LCMS	58.2 ± 16.5 49.5 ± 16.7	1.5 5.5	30.5 54.5	
Poh [167]	Indonesia Malaysia Thailand Vietnam	Boys and girls	276 861 495 384	0–12	CLIA	52.7 55.2 59.6 56.3	0 4.1 2.0 11.1	44 43.7 33.7 48.2	SEANUTS Nationally representative sample
Garg [168]	India 28.4°N	Adolescents Adult women and men	1829 1346	13.3 ± 2.8 58.0 ± 9.5	RIA	20.8 ± 13.0 24.5 ± 19.0	71.4 34.0	96.8 91.2	Delhi
Beloyartseva [169]	India	Male and female healthcare workers Multicenter study	2119	42.7 ± 6.8	RIA	35.9 ± 26.6		79	Physicians and healthcare professionals from 15 cities
Marwaha [170]	India 28.4°N	Pregnant women	541	24.6 ± 2.8	RIA	23.2 ± 12.2	59.5	96.3	Delhi
Shivane [171]	India 19.1°N	Young men Young women	558 579	25–35	RIA	47.2 ± 22.2 39.5 ± 22.7	12.0 26.4	62.0 76.1	Mumbai
Haugen [172]	Nepal 27°N	Young mothers–infant pairs (healthy lactating women)	500	25.8 ± 4.2 (17–44) Infants: 7 months (IQR 4–9)	LCMS	47.4 ± 16.4 82.0 ± 21.4	14.0 (<30) 0.6 (<30)	59.8 3.6	Bhaktapur municipality
Gromova [173]	Kazakhstan	Healthy adults Population-based	1347	44 ± 14	CMIA	Median range 27.5–45 between regions	27.5	69.9	Random sampling from six different regions, Vitamin D Standardization Program
Mehboobali [174]	Pakistan 24.9°N	Women Men	507 351	32.8 ± 10.1 32.1 ± 11.5	ECLIA	42.3 ± 17.2 60.1 ± 19.3		76 33	Karachi Low income
Junaid [175]	Pakistan 31.6°N	Women	215	28.4 ± 7.2	EIA	40.4 ± 34.4	43	73	Lahore

**TABLE 54.6** Vitamin D status and prevalence of vitamin D deficiency in Africa according to different studies.

References	Country Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D			Comments
						Mean ± SD nmol/L	<25 nmol/ L %	<50 nmol/ L %	
El Maataoui [178]	Morocco	Women >50 years Men >50 years	186 68	59.0 ± 8.2 64.7 ± 8.6	ECLIA	50.3 ± 23.3 51.8 ± 16.6	8.6 4.4		
Skalli [179]	Morocco 34.0°N	Adults Case–control study	146	33.6 ± 11.3	LCMS	32.5 ± 16.5		84.2	
Sherief [180]	Egypt 32.5°N	Children 14–18 years	572	17.6 ± 0.7	RIA	28.5 ± 13.3		94.8	Sharkia governorate
Aly [181]	Egypt 31.8	Men and women >60 years	176	67.7 ± 6.7	EIA	64 ± 9.5			Rural population in Dhakalia
Drali [182]	Algeria 36.7°N	Children 9 months–5 years	1016	3.0 ± 0.2	ELFA	46.5 ± 26.0	15.0	60.3	Municipality of Hussein Dey
Djennane [183]	Algeria 36.7°N	Schoolchildren		10 (8–13)	ECLIA	Median (IQR)			Tizi-Ouzou
		September	435	Median (IQR)		71.4 (48.2–79.5)	8.1	29.9	
		March	411			52.9 (39.4–75.6)	17.4	41.1	
Ben Fradj [184]	Tunesia 36.8°N	Adults Case–control study	225	63.3 ± 11.9	CLIA		34.8	62.4	
Faid [185]	Lybia 32.4°N	Men Women	75 380	33 (range 1–64)	ECLIA		29.3 (<30) 60.5 (<30)	52.0 79.4	
Jones [186]	Gambia 13°N	Pregnant women (first trimester), intervention study	863	29.6 ± 6.7	LCMS	70.2 ± 15.3	0 (<30)	7.4	DEQAS
Wakayo [187]	Ethiopia 8.3°N	Urban students	89	11–18	LCMS	48.2 ± 14.0		61.8	25(OH)D < 50 nmol/L Christians 35% Muslims 68.6%
		Rural students	85			61.0 ± 15.1		21.2	
Gebreegziabher [188]	Ethiopia 7°N	Women	202	30.8 ± 7.8	ELISA		14.8 (<30)	84.2	
Ghadegesin [189]	Nigeria 6.6°N	Pregnant women	461	31.3	HPLC	128		29	Lagos
Olayiwola [190]	Nigeria 7.4°N	Adults	120	>60	HPLC		51.4		Older Yoruba in Ibadan
Houghton [191]	Kenya 2°S	Preschool children			LCMS	Mean (95%CI)			Emali city
		Masaai	212	4.2 ± 1.1		95.7 (93.1, 98.3)		0	
		Kamba	285	4.3 ± 0.9		82.5 (80.7, 84.3)		1	
Friis [192]	Tanzania 2.7°S	Healthy adults	355	<25 to >55	CLIA	84.4 ± 25.6		4.3	Case-control study in Mwanza
		Tuberculosis pt	1223			110.9 ± 35.7		2.5	



Luxwolda [193]	Tanzania 2-4°S	Nonpregnant adults Pregnant women	88 139	33 ± 10	LCMS	106.8 ± 28.4 138.5 ± 35.0	0	1	Traditional outside lifestyle
Sudfeld [194]	Tanzania 6.8°S	Newborns Cohort from RCT	581	6 weeks 6 months	HPLC-MS	36.3 ± 18.5 65.0 ± 21.8		76.4 21.2	Dar es Salaam, longitudinal assessment
Mogire [195]	Kenya 3°S	Children (0–8 years)	1361	19.8 months	CLIA	81 (66–102)	0.3	6.0	
	Uganda 0°S		1301	24.1 months		79 (65–95)	0.4	5.1	
	Burkina Faso 11°S		329	23.4 months		78 (65–91) 71 (59–84) 76 (60–92) Median (IQR)	0	6.1	
	Gambia 13°S		629	46.6 months			0.3	9.9	
	South Africa 26°S		889	12.0 months median			1.9	13.5	
Durazo-Arviso [196]	Ghana 6°N	Adults 25–45 years	207	34.6 ± 6.7	LCMS	75.9 ± 6.9	0.2	4.8	Kumasi, Victoria, and Cape Town
	Seychelles 4°S		230	36.5 ± 5.1		72.9 ± 19.5	0	8.3	
	South Africa 34°S		232	33.7 ± 5.6		59.2 ± 20.7	6.6	34.1	
Valaphi [197]	South Africa 26°S	Pregnant women Newborns cord blood	291 291	28 ± 6	CLIA	57.0 ± 29.7 41.9 ± 21.0	16 (<30) 22 (<30)		Johannesburg
George [198]	South Africa 26.3°S	Pregnant women	343	29.9 ± 6.0	HPLC	41.9 (95%CI 21.8 –77.0)	35.8 (<30)	57.8	Soweto
Sebati [199]	South Africa 23.7°S	Young adults (20–29 years)	631	25.6 ± 2.0	RIA		96.6		Ellisras community
Lategan [200]	South Africa 29°S	Adults 25–64 years	339	44.3 ± 10.6	CLIA	38.4 ± 11.2	0	4	Bloemfontein, Blood sampling in autumn
George [201]	South Africa 26.2°S	African Asian Indian	373	41.6 ± 13.1	HPLC	70.9 (51–95)	3 (<30)		Johannesburg
			344	43.5 ± 12.9		41.8 (29–57)	15 (<30)		
Kruger [202]	South Africa	Black adults	179	<50	CLIA	77.3			North West Province
			298	50–60		71.2			
			129	60–70		66.2			
			52	>70		64.7			

### 3.7 Vitamin D status in Oceania

A selection of studies in Oceania, i.e., Australia, Pacific Islands, and New Zealand, is reported in Table 54.7. Latitudes in these studies range from 10° to 45°S. Although most countries in Oceania have a very sunny climate, a serum 25(OH)D concentration below 50 nmol/L is very prevalent, especially in pregnant Aboriginal women (56%) [205], community-dwelling men aged 70 years or over (43%) [209] and community-dwelling adults aged 50–80 years living in Tasmania (49%) [217], refugee children living in Sydney, Australia (61%) [210], and Northeast-Asian immigrants living in Canberra, Australia (68.8% in age group 30–39 years) [214]. Furthermore, low serum 25(OH)D concentrations (<50 nmol/L) were reported during early pregnancy (55%) [215], but less so in pregnant non-Aboriginal women in another study (20%) [205].

### 4. Multicenter and global studies using a central laboratory facility

Some studies have involved many countries or even several continents using one central laboratory facility. The advantage of these studies is that different assays for serum 25(OH)D and different laboratories are excluded as a source of variation. This is a great advantage because, as noted earlier, interlaboratory variation may be as high as 25% [7]. The Euronut Seneca study was done in older persons in European countries from the Mediterranean to Northern Europe [220]. In this study, there was a positive correlation between serum 25(OH)D and latitude, i.e., higher values in northern countries, the inverse of what was expected by sunlight exposure. This was confirmed by the baseline data of the MORE study, a study on the effect of raloxifene (a selective estrogen receptor modulator or SERM) versus placebo in postmenopausal women with osteoporosis [221], and baseline data of the randomized clinical trial with the SERM bazedoxifene [222]. In the latter (bazedoxifene) study, the correlation between serum 25(OH)D and latitude in other continents was negative as should be expected. The baseline data of the bazedoxifene study showed also a relationship between serum 25(OH)D and affluence with lower 25(OH)D levels in Eastern Europe than in Western and Northern Europe. The MORE study, the bazedoxifene study, and another global study [221–223] were all done in postmenopausal women with osteoporosis. Vitamin D status in these studies usually was better than in other studies because women participating in clinical trials usually are more concerned about their health. These three studies show a very poor vitamin D status in Middle Eastern countries confirming the data of national studies. Recently, values

from different European countries were standardized by the ODIN study [11]. The studies included in the European ODIN study usually are nationally representative and therefore a better estimate of the actual situation at least in Europe.

## 5. Ethnicity/migration

A recent review on vitamin D status in non-Western immigrants and refugees showed that vitamin D deficiency is common in this population [224]. Vitamin D status in immigrants from non-Western countries was poor in North America, Norway, the Netherlands, and Australia [21,114–116,225,226]. A review on this subject concluded that serum 25(OH)D in non-Western immigrants in the Netherlands was much lower than in those born in the Netherlands and was also lower than in people in their country of origin [227].

## 6. Nutrition

In Europe, a north–south gradient was observed for serum 25(OH)D with higher levels in Scandinavia and lower levels in Southern and Eastern European countries [220,221]. This indicates that other determinants than sunshine are of importance, e.g., nutrition, food fortification, and supplement use. Fortification of dairy products is practiced in the United States where vitamin D 400 IU is added per quart of milk. Fortification of milk is now also practiced in Sweden, Finland, and Ireland and resulted in a large improvement in vitamin D status in Finland [88].

## 7. Risk groups

Vitamin D deficiency is very common in certain risk groups, such as children with low birth weight (premature and small for gestational age), pregnant women, older people, and non-Western immigrants. Vitamin D status can be poor in adolescents as is seen in studies in Europe, the Middle East, and Asia [11,91,113,131,228,229]. Pregnant women, especially non-Western pregnant women and their children, are at high risk of vitamin D deficiency [116]. The dermal synthesis of vitamin D decreases with age, and especially older nursing home residents who do not come outside frequently are at high risk. Non-Western immigrants migrating to countries at higher latitudes with limited UV-B irradiation are at high risk because of more pigmented skin, the habit to stay out of the sun, the wearing of well-covering clothes, and a diet low in dairy products [114–116,226,227].

**TABLE 54.7** Vitamin D status and prevalence of vitamin D deficiency in Australia and New Zealand according to different studies.

References	City, state Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D		Comments	
						Mean ± SD nmol/L	<25 nmol/L %		
Malacova [203]	Representative sample of Australia	Adults 25+	5034	6 age groups	LCMS		4.5 (<30)	20.1	VDSP/NIST, highest prevalence in youngest age group (24–34 years) and in persons born in another country than Australia
Black [204]	Nationally representative sample of Aboriginal and Torres Strait Islander adults, 10°S	Adults 18+	3250	6 age groups	LCMS		4.7 (<30)	27	Prevalence higher in remote areas
Willix [205]	Kalgoorlie, Australia 31°S	Pregnant Aboriginal Pregnant non-Aboriginal	100 100	24.8 ± 6.2 29.5 ± 5.1	RIA	46.7 ± 21.7 65.4 ± 18.4	18 2	56 20	
Black [206]	Perth, Australia 31°S	Adolescents	1045	14 17	EIA LCMS	86 ± 27 75 ± 24		4.4 12.2	Good agreement for 12 samples between EIA and LCMS. Same participants at age 14 and age 17. Deseasonalized 25(OH) D
Mulrennan [207]	Shire of Busselton, Western Australia 33°S	Caucasian adults aged 45–69 years	5011	58.0 ± 5.8	CLIA	81.3 ± 25.9		8	117 samples also assessed using LCMS, correlation of 0.94
Ke [208]	Sidney, Australia 33°S	Children (longitudinal): - 8 years - 15 years	249 162		RIA	94 ± 25 63 ± 16			25(OH)D analyses at 2 time points. 25 matched pairs reassayed in 1 batch. At age 15, girls on average 6 nmol/L lower 25(OH)D than boys
Hirani [209]	Sidney, Australia 33°S	Community-dwelling men 70+	1659	4 age groups	RIA	55.9 ± 22.2	9.6 (<30)	43	
Sheikh [210]	Sidney, Australia 33°S	Refugee children attending an outpatient general health clinic	215	0–17	RIA	46 ± 24	21	61	Majority (76%) from Africa
Gill [211]	Adelaide, Australia 34°S	Adults	2413	50.6 ± 16.6	EIA/CLIA	69.2 ± 26.4	0.9	22.7	
Daly [212]	Australia 30–35°S	Population-based random sample of adults	11,218	≥25	CLIA	62.8 ± 25.4	4	31	Correlation of 0.85 in 70 samples when compared with LCMS.

**TABLE 54.7** Vitamin D status and prevalence of vitamin D deficiency in Australia and New Zealand according to different studies.—cont'd

References	City, state Latitude°N/S	Study population	N	Age Years	25(OH)D method	25(OH)D		Comments	
						Mean ± SD nmol/L	<25 nmol/L %		
Zhou [213]	Adelaide, Australia 35°S	Representative population sample of preschool children	221	1–5	RIA	73 ± 26	4 (<30)	16 (30–50)	Royal College of Pathologists of Australasia and Australasian Association of Clinical Biochemistry (RCPA/ AACB) Quality Assurance Program (QAP) Higher prevalence in winter (8% and 22%)
Guo [214]	Canberra, Australia 35°S	Northeast-Asian immigrants	43 17 21 19	18–29 30–39 40–49 50–80	LCMS	58.6 ± 23.6 46.3 ± 21.2 58.7 ± 18.9 76.8 ± 21.0		38.5 68.8 30.0 10.5	DEQAS. Total sample: mean 25(OH) D = 60.2 ± 23.5; 36% < 50 nmol L; 3% < 25 nmol L
Davies-Tuck [215]	Victoria, Australia 36°S	Early pregnancy	1550	30.0 ± 5.4	CLIA	47.0 (range 12–178)		55%	Women with 25(OH) D < 75 nmol/L were recommended to take 1000 IU/d and had an additional measurement at 28 weeks
Nessvi [216]	Auckland 36°S and Dunedin 45°S, New Zealand	Multiethnic sample of adult volunteers	133 121 130 119	18–34 35–49 50–64 65–85	LCMS	45.2 (SE 1.8) 44.6 (SE 2.0) 52.0 (SE 1.9) 51.3 (SE 2.0)			
Thompson [217]	Southern Tasmania 42°S	Random sample of community-dwelling adults aged 50–80 years	1096	62.5 ± 7.5	RIA	Baseline (2002–04): 52.2 ± 17.0 (deseasonalized)		49	Quality assurance program, assay validated against LCMS. Mean 25(OH)D increased to 59.6 after 2.5 years and 63.5 after 10 years
Boyle [218]	Auckland, New Zealand 37°S	Pregnant women (15 weeks of gestation)	1710	30.3 ± 4.7	LCMS	72.9 ± 27.0	5%	23%	DEQAS
Polak [219]	Otago, New Zealand 45°S	Healthy university student volunteers	615	19.5 ± 1.5	LCMS	64.1 ± 26.6			External quality control serum material containing low and medium concentrations in every run

## 8. Trends in vitamin D status

General attention for widespread vitamin D deficiency may lead to recommendations for vitamin D supplementation and public health measures such as food fortification with vitamin D to improve vitamin D status [88]. On the other side, fear of skin cancer may increase the use of sunscreen, which may decrease vitamin D production in the skin [55]. Positive or negative trends in vitamin D status can only be reliably diagnosed when the measurement of serum 25(OH)D is free from bias. When two population samples with an interval of 5 or 10 years are compared, the serum 25(OH)D values should be made comparable by cross-calibration, using a gold standard assay, such as LC-MS, and DEQAS or preferably VDSP [11,14].

Trends were studied in the NHANES population study using LC-MS calibrated to a standard reference [56]. Serum 25(OH)D did not change between 1988 and 2006, but it increased 5–11 nmol/L between 2007 and 2010, the highest increase being observed in older white women probably due to supplement use. This was confirmed in the Study of Women's Health Across the Nation [57]. A negative trend was seen in Canadian children, probably due to decreased fish and milk consumption [58]. A negative trend was also seen in the Inuit population of Greenland between 1987 and 2005 [59,60]. Vitamin D insufficiency (serum 25(OH)D < 50 nmol/L) was observed in 77% of young adults. The decrease was attributed to the change of traditional diet of fish and sea mammals to a Western diet.

In the United Kingdom, an increase in the prevalence of rickets was seen in hospital discharge data [61]. Conversely, the free distribution of vitamin D3 400 IU/d to infants in Turkey led to the almost disappearance of rickets in infants and toddlers in a few years [118]. A very positive trend in vitamin D status was observed in Finland after the fortification of milk and yogurt with vitamin D3 400 IU/L [88]. Mean serum 25(OH)D increased 17 nmol/L between 2000 and 2011, and the prevalence of vitamin D deficiency decreased from 59% to 14%. Positive trends in vitamin D status were also observed in Lebanon and Iran [62,63]. These positive trends were attributed to more awareness, more screening, and more supplementation. A similar positive trend was observed in India, where the analysis of serum samples in a large hospital showed an increase of mean serum 25(OH)D and a decrease of vitamin D deficiency [64]. On the other side, a very significant decrease of vitamin D status was seen in South Korea between 2008 and 2014. Mean serum 25(OH)D decreased 10 nmol/L in men and 7 nmol/L in women [65]. The decrease was attributed to urbanization, air pollution, and less outdoor activity.

In conclusion, positive trends can be observed, mainly with vitamin D supplementation and food fortification, while negative trends are the consequence of dietary changes and less sun exposure.

## 9. Implications

Vitamin D deficiency has been classically associated with mineralization defects, bone loss, osteoporosis, and fractures [66,222] (see Chapters 62 and 63). The causal relationship between vitamin D deficiency and fractures has been confirmed by randomized clinical trials [67]. Vitamin D deficiency has also been linked to muscular weakness, decreased physical performance, and falls [68–70]. The latter relationship has also been confirmed by clinical trials [71]. In recent years, vitamin D deficiency has been associated with nonclassical outcomes, such as cardiovascular disease, diabetes mellitus, multiple sclerosis, tuberculosis, respiratory infections, and several types of cancer [72]. However, for all of these nonclassical outcomes, many clinical trials have been negative, and causality has not been established. The magnitude of the negative health effects attributed to vitamin D deficiency also depends on the percentage of the population having a low vitamin D status. Roughly 50% of the Western–European population has a serum 25(OH)D concentrations below 50 nmol/L at least in winter. This percentage is lower in North America and appears higher in South America. The prevalence of vitamin D deficiency is higher in Northern African countries and South Africa than in sub-Saharan Africa. Vitamin D deficiency is around 50% in Oceania. Studies from the Middle East and Asia reveal severe vitamin D deficiency in the Middle East, China, Mongolia, and India. It is important to do more research in these countries, especially in Asia, where a relatively large part of the world population lives.

In summary, to be able to estimate the burden of vitamin D deficiency, more prevalence studies are needed in Eastern Europe, the Middle East, Asia, Africa, and some South American countries. Quality control of the serum 25(OH)D assays should be done at least by participation in a quality assurance scheme such as DEQAS [8], and the % deviation from the overall mean should be reported. Preferably, results should be standardized by participating in a program such as VDSP [11].

Prevention requires moderate sunlight exposure, consumption of fish, fortification of foods with vitamin D, and the use of vitamin D supplements. A supplement of vitamin D3 400 IU/d can be recommended for children and also for adults who do not come outside or have a dark skin. Pregnant and lactating women may require 400–800 IU per day. Older persons also require



a supplement of 400–800 IU per day, the higher dose with insufficient sun exposure or dark skin. Patients with osteoporosis and older persons in rest or nursing homes require 800 IU per day. It will require an enormous effort to bring up serum 25(OH)D levels to more than 50 nmol/L in all continents all year long.

## 10. Conclusions

Vitamin D deficiency (serum 25(OH)D < 25 nmol/L) and low vitamin D status (serum 25(OH)D < 50 nmol/L) are very common in most countries around the world. Severe vitamin D deficiency is common in the Middle East, China, Mongolia, and India. Risk groups are children, especially those with low birth weight, adolescents, pregnant women, older persons, and non-Western immigrants. Probably less than 50% of the world population has an adequate vitamin D status (serum 25(OH)D > 50 nmol/L) at least in winter. Prevention requires moderate sun exposure, consumption of fish, fortification of foods, and the use of vitamin D supplements.

## 11. Summary points

- Vitamin D deficiency and low vitamin D status are very common in most countries around the world.
- More prevalence studies are needed in Eastern Europe, the Middle East, Asia, Africa, and some South American countries.
- Studies should participate in a quality control program such as VDSP to make prevalence rates comparable.
- Vitamin D deficiency (25(OH)D < 25 nmol/L) and low vitamin D status (serum 25(OH)D < 50 nmol/L) can be prevented by moderate sun exposure, consumption of oily fish, fortification of foods with vitamin D, and use of vitamin D supplements.

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# Vitamin D in food—Compounds, stability, sources

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## OBJECTIVES

- Describe the chemistry of vitamin D—provitamins and vitamers.
- Describe the chemical changes of vitamin D due to physical factors such as light, temperature, pH, and oxygenation.
- Outline the analysis of vitamin D in food.
- Describe the distribution of vitamin D in foods in nature (natural content).
- Discuss the effects of production facilities on vitamin D content in primary food.
- Discuss the chemical changes in vitamin D during food processing (retention).
- Detail the content of vitamin D in food as documented in databases.
- Discuss the calculation of dietary intake of vitamin D.

## 1. Introduction

Even though vitamin D is not a vitamin for all humans, as it depends on whether we have access to sun or not, the content in our food is essential. The recommendation for daily dietary intake of vitamin D is set to 10–20 µg/day [1–3]. Information on the food types in our diet will enable estimation if the dietary intake meets, or to what extent meets, the daily dietary recommendation for vitamin D.

One of the great scientists Sir Lord Kelvin said: “When you can measure what you are speaking about and express it in numbers, you know something about it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind.” This statement somehow makes sense for vitamin D in food. The discovery and elucidation of the understanding of the physiological mechanism of vitamin D are linked to the history of analytical chemistry, and this explains why it took 50 years to discover the active vitamin D forms, and why some of our knowledge of content of vitamin D in food is still limited.

Cod liver oil was part of the discovery of vitamin D [4]. This was followed by investigation of the effect of sunlight on baby chicken and pigs [5,6], as well as sunlight and artificial light on cows and milk [7,8]. The irradiation resulted in a positive outcome for the animal husbandry and the food produce, which enabled the statement in 1925: “the time is probably not far distant when every producer of high grade milk will find it necessary to irradiate his cows artificially” [7]. The researchers observed a positive effect in curing rickets, but did not have the tools (analytical methods), to estimate changes in vitamin D. In the 1920–1960s, the analytical tools to understand vitamin D in food or as a pure compound were limited to biological methods, which could estimate the vitamin D content by their effect in curing rickets in animals, or spectrophotometric changes to evaluate the effect of exposure of light [9–11].

A lot of effort has been put into understanding the degradation of vitamin D (crystals or in solution). The knowledge of the effect of physical factors like light, temperature, pH, and oxidation on vitamin D, was mainly established before 1980. In the 1970s, the breakthrough of liquid chromatography eventually enabled

the development of methods, which could quantify the level of vitamin D ( $\mu\text{g}/100\text{ g food}$ ) [12,13]. Then in the 1990s the principle for the method, which is used today, was introduced and the content in foods on the market (in Finland) of the specific vitamin D vitamers was stated [14]. Vitamin D is presented in food in an amount of  $\mu\text{g}/100\text{ g food}$  [15]. Statements of where to find vitamin D in our food usually include fatty fish, eggs, organs, and meat, and if available, fortified food, but neglects the information that vitamin D stability is sensitive to oxygen and light.

The aim of this chapter is to describe the physical factors that induce vitamin D degradation; the analytical methods used to quantify vitamin D in foods; natural, current, and potentially future content in foods; the effect of storage and processing; quantification of total vitamin D activity; challenges of using food composition data; and then consider the prospects for future research.

## 2. Chemical structure of vitamin D vitamers

### 2.1 Which chemical structures have vitamin D activity?

The provitamin D, i.e., the sterols, 7-dehydrocholesterol (7DHC), and ergosterol, has the steroid form (ring A, B, C, D), see Fig. 55.1. The vitamin D compounds are a family of 9,10-secosteroids, which for the parent form differ only in side-chain structure. Compared to sterols, secosteroid has an open ring, which for vitamin D is the B-ring. The difference between the primary vitamin D compounds, cholecalciferol (vitamin D<sub>3</sub>) and ergocalciferol (vitamin D<sub>2</sub>) is a double-bound in the side chain between carbon 22 and carbon 23, and the extra methyl group at carbon 24,

see Fig. 55.1. The intermediate previtamin D is a 6,7-cis isomer form and isomerize in a temperature-dependent equilibration to vitamin D [16,17]. In animal products, the vitamin D vitamers 25-hydroxyvitamin D (25(OH)D) and 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) are present due to the metabolism in vertebrates i.e., 25-hydroxylation and 1 $\alpha$ -hydroxylation primarily in the liver and the kidneys (see Chapters 4, 8 and 9) [18].

Besides vitamin D<sub>2</sub> and vitamin D<sub>3</sub> other parental forms of vitamin D vitamers are vitamin D<sub>4</sub> to vitamin D<sub>7</sub>, see Table 55.1. However, the interest of these vitamers in food is limited, due to their insignificant biological activity, which has been investigated for vitamin D<sub>4</sub> and vitamin D<sub>5</sub> [19–21]. Similarly, the dihydroxylated metabolites e.g., 1,25(OH)<sub>2</sub>D and epimers of 25(OH)D will in general not be covered.

## 3. Stability of vitamin D

### 3.1 What can happen to the antirachitic compounds vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in crystal form and when dissolved in organic solvents?

Knowledge of the stability of vitamin D is especially important to take into consideration when analyzing (extraction and sample clean-up), quantifying vitamin D in different matrixes, and when storing samples and standards. These processes are further important to comprehend to understand and avoid the degradation of vitamin D during food processing and storage.

Vitamin D was differentiated from vitamin A as cholecalciferol is more heat stable and resistance to oxidation. The observation was that cod liver oil heated (120°C) and aerated for 12 h still has the ability to cure rickets, while not able to cure xerophthalmia in rats

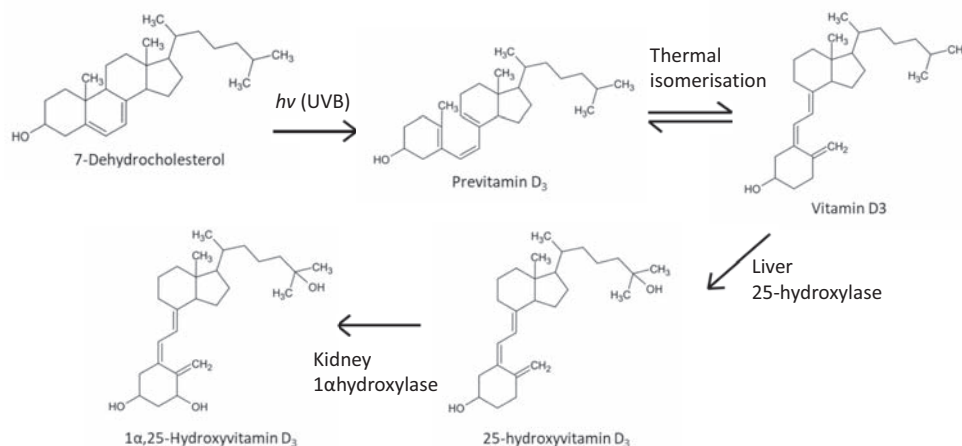
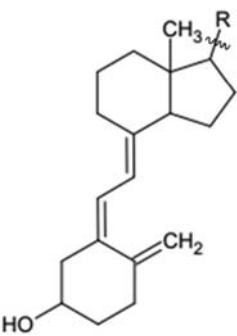
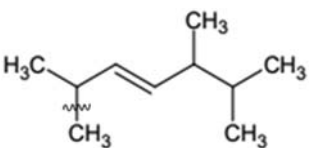
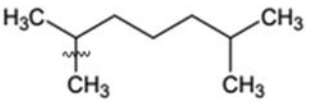
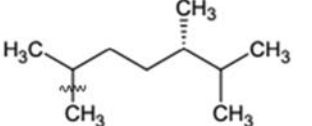
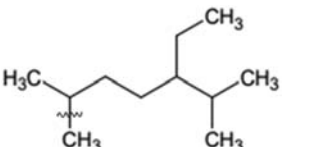
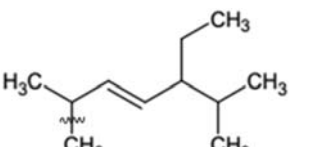
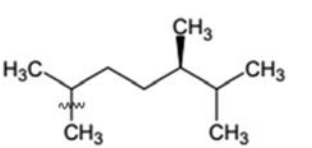


FIGURE 55.1 Metabolism of vitamin D in vertebrates.

TABLE 55.1 Structure of the different vitamin D vitamers with their specific provitamins.

	R	Vitamin D	Provitamin
		D2	Ergosterol
		D3	7-Dehydrocholesterol
		D4	22,23-Dihydroergosterol
		D5	7-Dehydrositosterol
		D6	7-Dehydrostigmasterol
		D7	7-Dehydrocampesterol

[4]. Studies into the synthesis, stability, and analyses of vitamin D have identified the physical factors and the degradation products from exposure to light (different wavelength), temperature, pH, and oxidation.

### 3.2 Exposure to light

Ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>) are produced by UVB-exposure to ergosterol and 7DHC [22,23]. Further exposure of ergocalciferol by 247 nm up to 290 nm in ethanol and of crystals showed loss of antirackitic effect [24]. The compounds identified were toxisterols generated from previtamin D and suprasterol I and II from vitamin D [25,26], see Fig. 55.2A. Sunlight exposure of vitamin D<sub>3</sub> in methanol revealed formation besides suprasterol I and II, also 5,6-trans vitamin D<sub>3</sub> [27]. Thirteen toxisterols have been isolated, and the structure of compounds depends on the solvent in which they are formed e.g., alcohol or ether [25]. Vitamin D chromophore cause

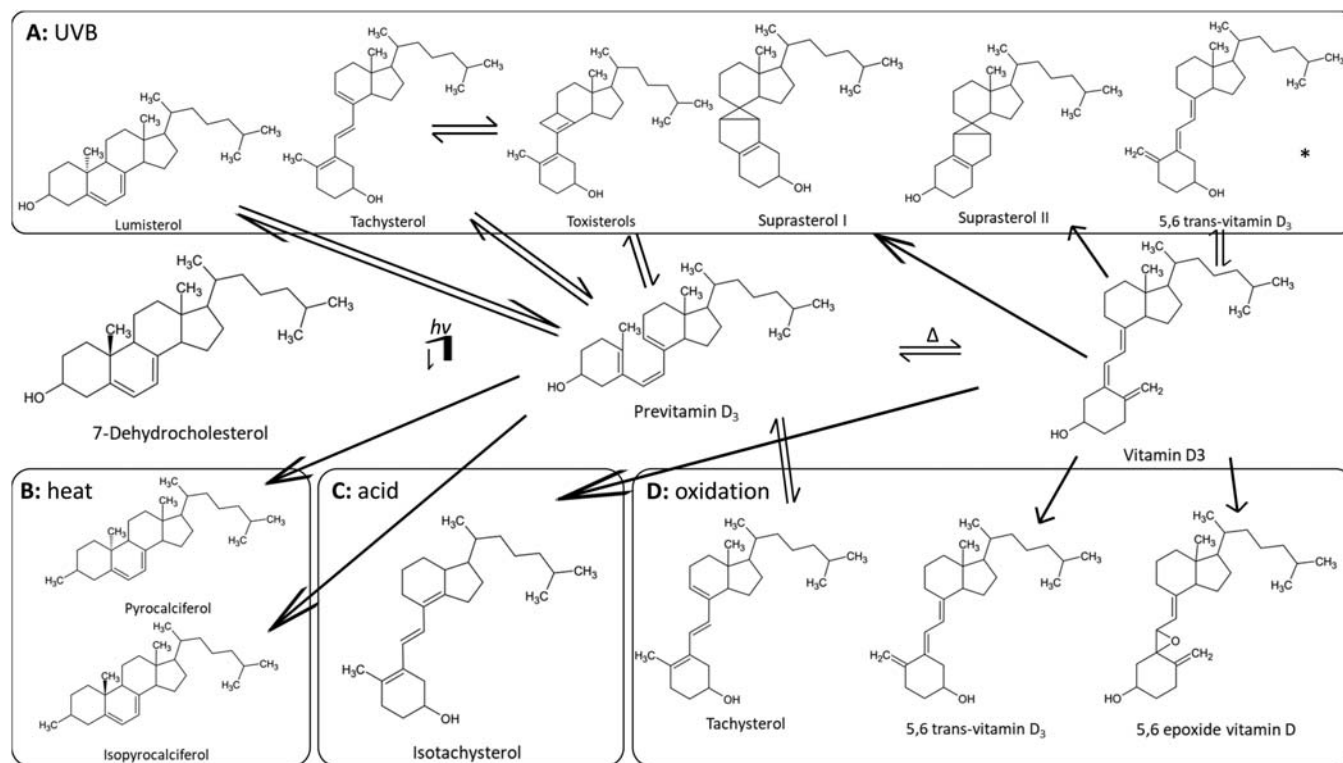
the absorption of light in the same wavelength as provitamin D (7DHC) and previtamin D. Thus the photochemical transformation of these compounds takes place under the same environmental condition (wavelength of light exposure) [28].

Crystals of vitamin D<sub>2</sub> degrade in amber glasses, but to a lesser extent than in ordinary glasses [11]. A solution of vitamin D<sub>3</sub> in acetonitrile showed no degradation by indoor light (fluorescent lamp F15T8/CW) for 10 days [29]. Solutions of vitamin D<sub>3</sub>, vitamin D<sub>2</sub>, and 25(OH) D<sub>3</sub> in ethanol in clear, glass vials stored either in dark for 7 days, exposed to light (no UVB and UVA) for 7 days, or exposed to UVB for 7 days, showed significant degradation when exposed to UVB ( $P < .01$ ) for all three vitamers (Jakobsen, personal communication).

### 3.3 Thermal treatment

Thermal degradation was shown for ergocalciferol at temperatures above 150°C during distillation, and





**FIGURE 55.2** Vitamin D isomerization products formed by UVB-exposure (A), \* identified following sunlight exposure; thermal treatment above 150°C (B); acid (C), the primary product at low temperature; oxidation/singlet oxygen (D). Exemplified by vitamin D<sub>3</sub>.

the resulting compound was termed pyrocalciferol [23,24], while isopyrocalciferol was designated at a later stage [30,31]. Heating experiments of cholecalciferol identified the thermal products to derive from the precholecalciferol [32]. See Fig. 55.2B. Heating cholecalciferol in different solvents found the thermal isomers formed from 100 to 170°C, while in a model system the degradation is estimated to start at 156°C and 166°C for vitamin D<sub>3</sub> and vitamin D<sub>2</sub>, respectively [32,33].

### 3.4 Treatment at different pH

Treatment of vitamin D by acid results in the isomerization of previtamin D to primary isotachysterol and some isocalciferol, and the isomerization rate depended on acidity [34]. Acid isomerization of vitamin D<sub>3</sub> in methanol in the dark at ambient temperature produce isotachysterol, but also seven oxygenation products in a self-initiated autoxidation reaction [35]. However, only isotachysterol is formed at low temperature (0°C), in the dark, and under inert gases such as nitrogen. This procedure was applied in a gas chromatographic method [36]. See Fig. 55.2C. The stability toward alkaline pH was not essential until alkaline saponification (15% KOH in methanol) was investigated as a procedure to remove fat from the vitamin D matrix. High pH did

not cause any degradation of vitamin D<sub>3</sub>, which was used for the analyses of vitamin D by liquid chromatography [37].

### 3.5 Oxidants and singlet oxygen

In the presence of the oxidant iodine previtamin D isomerizes to 5,6 trans previtamin D (tachysterol), while vitamin D isomerizes to 5,6 trans vitamin D. See Fig. 55.2D. The higher temperature the equilibrium between previtamin D<sub>3</sub> ↔ vitamin D<sub>3</sub> is favorable for previtamin D<sub>3</sub>. Thus at room temperature 5,6 trans vitamin D is formed, and at temperatures such as 70°C, also tachysterol [31,38].

Vitamin D<sub>3</sub> in a solution of methanol and acetonitrile and exposed to a stream of oxygen or air showed degradation within 1.5 h and over a period of 10 days [29,37].

The singlet oxidation of the conjugated triene part of vitamin D<sub>3</sub> was initially generated by sodium hypochlorite-hydrogen peroxide in a vitamin D<sub>3</sub> solution [39]. Vitamin D oxidation with singlet oxygen formed vitamin D epoxides [40,41]. Riboflavin, a water-soluble vitamin, is an excellent photosensitizer if exposed to light. In a solution with vitamin D and exposed to light, riboflavin produced singlet oxygen, which oxidized vitamin D. The oxidation of vitamin D

did not occur if not light exposed [42]. One of the compounds formed is 5,6-epoxide of vitamin D [43]. See Fig. 55.2D.

### 3.6 Solvents and vitamin D

Crystal powder of vitamin D<sub>3</sub> could be stored under nitrogen or in vacuum at 25°C for up to 56 days, while vitamin D<sub>2</sub> in vacuum start degrading after 7 days [44]. The stability of vitamin D at lower temperatures depends on its solvent e.g., vitamin D<sub>2</sub> and vitamin D<sub>3</sub> are stable for up to 36 months in propylenglycol and amber vial, without replacement of air [45]. In isooctane or ethanol in vacuum or under nitrogen revealed that no loss was observed in ethanol, but loss appeared in isooctane [37]. The stability of vitamin D<sub>3</sub> in acetonitrile at 4°C versus 21°C for a period of 10 days, was significantly affected by temperature, with significantly higher stability at 4°C [29]. Storage at –20°C for crystals and in ethanol or n-heptane for a minimum of 2 years can be done without any significant degradation of vitamin D vitamers (Jakobsen, personal communication). Table 55.2 sums up the environmental factors, which cause vitamin D to become unstable or stable as crystals or in solution.

## 4. Analytical methodology

### 4.1 Principle for the extraction, detection, and quantification of vitamin D (vitamin D<sub>3</sub>, 25(OH)D<sub>3</sub>, vitamin D<sub>2</sub>, and 25(OH)D<sub>2</sub>) in foods including LOQ, precision, and accuracy

Vitamin D is present in free form but exists also in other forms, for example as fatty acid esters [46,47]. Vitamin D is stable in an alkaline solution, and alkaline saponification is the preferred method for the degradation of fat in food samples. The analytical method to quantify vitamin D vitamers in food includes steps

similar to those introduced 50 years ago: alkaline saponification, followed by liquid-liquid extraction followed by a selection of clean-up steps, which depend on the final detection and quantification principle. For vitamin D in food, the standard methods for vitamin D [48] include a solid-phase extraction, semipreparative HPLC and/or thin layer chromatography, before the final detection by DAD/UV, and quantification by use of internal standard for vitamin D<sub>2</sub> for vitamin D<sub>3</sub> and vice versa. The original establishment of certified reference materials for the content of vitamin D revealed that the analyst needs to be skilled and ensure correct sampling in the preparative clean-up, a solvent suitable to dissolve the vitamin D, and a satisfactory baseline resolution in the final analytical chromatography [49]. The same principle was introduced for quantification of 25(OH)D [14]. The development of tandem mass spectrometry methods to quantify vitamin D and 25(OH)D in food diminished the number of clean-up steps to primarily alkaline saponification, liquid-liquid extraction, and an SPE-step before injecting the solution to the LC-MS/MS. The use of Diels-Adler derivatization step to form a vitamin D adduct which enhances the signal using electrospray ionization was initially introduced for analyses in plasma and then transferred to food [50,51]. This principle is used in the standard method for vitamin D in infant formula and adult nutritional [52], while no standard method is available for 25(OH)D either using DAD/UV-detection or tandem mass spectrometry.

Pitfalls in the analyses for vitamin D in food include the fact that deuterium labeled standards do not necessarily compensate for ion suppression or ion enhancement during ionization as the deuterated form elutes earlier, thus carbon labeled vitamin D standard should be prioritized [53]. Furthermore, the mandatory use of internal standards is caused by the equilibrium between previtamin D ↔ vitamin D [16]. In food, no difference appears between saponification at room temperature (20°C) and at high temperature e.g., 75°C [14,54].

**TABLE 55.2** condition leading to instability or stability of vitamin D<sup>a</sup>.

Factor	Vitamin D (crystals and pure solution)		Vitamin D in foods	
	Instable	Stable	Instable	Stable
Light	UVB	x (>UVA)	UVB (>content)	x (>UVA)
Temperature	>150°C	–20°C	>150°C	–20°C
PH	Acid	Neutral, alkaline	n.o.	Neutral, alkaline
Oxidation	X		X	
Singlet oxygen	X		x	

<sup>a</sup>Presence of water i.e., humidity/water activity accelerates loss.  
n.o. no observation.

However, in the analysis of fortified foods the analyst should be aware of the potential risk that internal standards do not fully correct for equilibrium. In this case, hot saponification should be prioritized [55].

The reliability of data for the content of vitamin D in foods is dependent primarily on documentation of trueness, precision, and the limit of quantification. Trueness describes the quantified level compared to other laboratory or referenced to certified reference material. The challenge for vitamin D research is the limited number of reference materials with certified values [56], and that a proficiency test is only available for vitamin D<sub>3</sub>. Precision describes the within laboratory repeatability and between laboratory repeatability (reproducibility) and is assessed during the standardization of a method, which for vitamin D<sub>3</sub> in food [48] is based on data from certification of e.g., fortified margarine showing 6.5% and 8.4%, respectively [49]. The validation of the LC-MS/MS standardized method for infant formula and adult nutritional included eight laboratories, which used the standardized method description in the analyzes of samples (0.7–14 µg vitamin D<sub>3</sub>/100 g). The repeatability and reproducibility obtained were max. 5.8% and 12.7%, respectively [52]. No standardized method is available for 25(OH)D<sub>3</sub> in food, but to provide an idea of the precision for this vitamin the results from a laboratory comparison (five laboratory using own in-house LC-MS/MS methods) analyzed samples containing >0.1 µg/100g. Repeatability and reproducibility were for vitamin D<sub>3</sub> max. 9.7% and 14.8%, respectively, and for 25(OH)D<sub>3</sub> max. 16% and 24%, respectively [57]. It is expected that at lower levels a higher repeatability and reproducibility is observed, especially if no optimization was done beforehand [49].

The capability of an analytical method is also described by the limit of quantification (LOQ), which is essential for vitamin D. The method chosen should fulfill the criteria for the lowest content of interest. If values are intended for food databases, the LOQ preferable should be defined based on the expected dietary intake. The optimal analysis will be that which is performed accredited according to ISO17025, which e.g., ensures the documentation of trueness and uncertainty. For the studies referred to in the next sections, the information on trueness is in the range 80%–120% and repeatability <10% [14,54,58–61]. Further details on vitamin D assay standardization in the analyses of serum/plasma are provided in Chapter 49.

In the description of analytical methods, biological methods used for the analysis of food were by choice omitted. Thus, in the forthcoming section only data derived from specific, chemical methods are included. In the investigation of cause and effect, repetition is essential, and studies are only included if a minimum

of three replicates and precision are available or can be calculated from data in the original paper.

## 5. Vitamin D in food

Generally, only foods of animal origin contain vitamin D, and from the early history of vitamin D research, it was observed that sunlight has an enormous effect on the produce of antirachitic compounds. Thus, the vitamin D content of animal products is controlled by biological metabolism and may vary with the amount of vitamin D in animal feed, access to pasture, season, and between animal species. The majority of animal husbandry involves living indoors. In this section only results reported from free-living animals are reported while the content in farmed animals/aqua-cultured fish is reported separately—and in combination with the possibility for biofortification. All content is presented as µg/100 g fresh weight. Recalculation from IU to µg by multiplication 0.025 has been done. If data is presented per g dry matter (DM) value was recalculated to per g fresh weight, and if % DM is not presented the assumption of the content of % DM is indicated in each case.

### 5.1 Vitamin D in food from wild fish and animal

The natural level of vitamin D in fish and animals is difficult to define and assess, partly because specific data is lacking due to the analytical challenges, and partly because the specification of the analyzed sample not necessarily is reported. For example, salmon may be a *Salmo salar* or a *Onchorhynchus* sp. The specific challenges will be discussed for each of the possible vitamin D sources, including fish, egg, meat, organ, mushroom, plants, and insects.

It was noted that fish liver oils contained varying amounts of vitamin D, up to 112,500 µg/100 g from oriental tuna, 17,500 µg/100 g from swordfish, 3000 µg/100 g from halibut, and 2500 µg/100 g from Atlantic cod [9]. By exception, these values are based on the biological method, but similar high values or even higher were reported by a chemical method e.g., halibut liver oil at 9200–13,000 µg vitamin D/100 g and in cod liver oil 8800 µg vitamin D<sub>3</sub>/100 g [13]. Similarly, huge variation of vitamin D in flesh from wild-caught fish species has been shown in the range of 1–27 µg vitamin D<sub>3</sub>/100 g [58,62]. The content of vitamin D is not correlated to the content of fat, age, weight or sex, but an effect of habitat has been shown [58,62–64]. Atlantic salmon (*Salmo salar*) when caught in the Baltic Sea is reported to have a content of 18.5–26.5 µg vitamin D<sub>3</sub>/100 g, if caught in the North Sea/Atlantic Ocean the content is

reported to 8.4–9.4  $\mu\text{g}$  vitamin D<sub>3</sub>/100 g [58,63,65]. Wild salmon caught in the Pacific Ocean (*Onchorhynchus* sp) shows a content of 14.2  $\mu\text{g}$  vitamin D<sub>3</sub>/100 g [66]. The variety of vitamin D in salmonids nicely demonstrates the huge variation which nature provides us. See Fig. 55.3, which shows the content of vitamin D and fat in selected fish species. Within a fish species, the sampling of herring and mackerel over a year shows the independence of vitamin D from levels of fat within a species, see Fig. 55.4.

In addition to the content of vitamin D in fish, the origin of vitamin D in fish has been investigated. In the early days of vitamin D research estimation of vitamin D in phyto- and zoo-plankton was hampered by sensitivity and specificity of the analytical method available. The conclusion was no vitamin D in phyto-plankton, but minor in zoo-plankton [67]. In the last 30 years, a few studies did report the natural level of zoo-plankton from lakes in Japan and in Finland, and from the Indian Ocean to approx. 0.015–2.7  $\mu\text{g}$  vitamin D<sub>3</sub>/100 g

(assuming 1% DM [64]) [64,68,69]. While the content of vitamin D<sub>3</sub> in phyto-plankton from a Japanese lake and the Indian Ocean contained approx. 0.22–8.0  $\mu\text{g}$  vitamin D<sub>3</sub>/100 g (assuming 10% DM) [68,69].

The natural level of vitamin D in eggs can be hypothesized to be the level in eggs from hens, which in a research project was provided for hens who had the possibility to stay outside for a period of 4 weeks in summertime (51°N). The content in these eggs was 4.5  $\mu\text{g}$  vitamin D<sub>3</sub>/100 g and 1.3  $\mu\text{g}$  25(OH)D<sub>3</sub>/100 g [70].

In contrast to hens, dairy cows have often access to pasture, and dairy cows delivering organic milk, they have access to pasture from spring to autumn. For dairy cows sun-exposed during summer in New Zealand (38°S) and in Denmark (55°N), the maximum content of vitamin D<sub>3</sub> in milk fat per 100 g was 0.58 and 0.53  $\mu\text{g}$  vitamin D<sub>3</sub>, respectively [61,71]. These levels indicate a level in fresh, whole milk (3.5% fat) of approx. 0.020  $\mu\text{g}$  vitamin D<sub>3</sub>/100 g, and 0.008  $\mu\text{g}$  25(OH)D<sub>3</sub>/100 g [71].

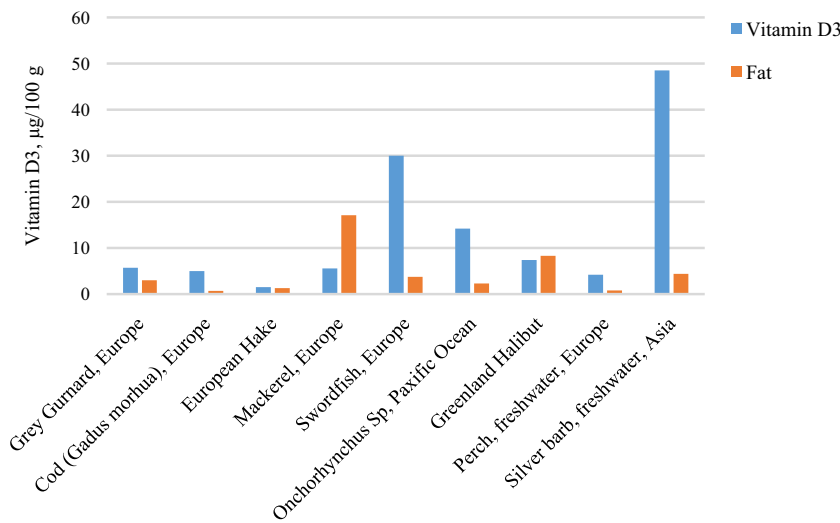


FIGURE 55.3 Content of vitamin D and fat in fish species from natural habitats [58,64,65,89,145].

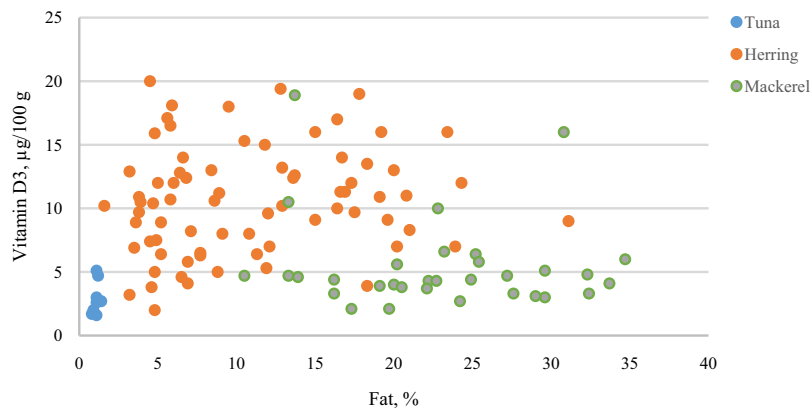


FIGURE 55.4 Content of vitamin D and fat in tuna, mackerel, and in herring wild caught.



For meat, the content of vitamin D in beef cattle on pasture in Queensland, Australia (17°S) indicates in minced beef (10% fat) a content of 0.24 µg vitamin D<sub>3</sub>/100 g and 0.11 µg 25(OH)D<sub>3</sub>/100 g [72]. For pork, the content of vitamin D from free-range pigs, which have mandatory access to outdoor areas in Denmark (55°N), showed a content of 0.88 µg vitamin D<sub>3</sub> and 0.38 µg 25(OH)D<sub>3</sub> per 100 g lean meat (4.2% fat) [73].

Wild-grown mushrooms in Sweden and Finland had a content of 10.7–29.8 µg vitamin D<sub>2</sub>/100 g *Cantharellus* and 2.9–58.7 µg vitamin D<sub>2</sub>/100 g *Boletus Edulis* [74,75], while analyses of wild grown white button mushroom (*Agaricus Sp.*) from Denmark and Finland did reveal content of 0.2–1.5 µg vitamin D<sub>2</sub>/100 g [75,76]. Otherwise, the knowledge of vitamin D in plants is limited, but in edible vegetables vitamin D<sub>3</sub> is not present [77]. Even though lichen is not a foodstuff for humans, it should be mentioned that this symbiotic organism, which consists of a fungus and an alga, has been found to contain vitamin D<sub>3</sub> in an amount of 2.3–4.7 µg per 100 g (assuming 10% DM) [78]. Future sustainability goals may include insects in the diet, which are reported to contain <0.01 µg vitamin D<sub>3</sub>/100 g (assuming 10% DM) [79].

## 5.2 Vitamin D in food farmed by man-fish, eggs, milk, meat (pork/beef/chicken), mushrooms, and plants

What are the levels of vitamin D in marketed food products, and what levels have been reported in research facilities? First of all, data in food composition tables are intended to provide the best estimate for the content of food on the market in the difference areas/countries of the world (<https://www.fao.org/infoods/infoods/tables-and-databases/faoinfoods-databases/en/>). However, few of these databases specify the individual content of vitamin D vitamers and published data for these individual results are limited, mainly due to the high cost of vitamin D analyses, and the relatively short time-frame in which analytical methods have been available. Thus, this section highlights the published results, which have reported the content of individual vitamin D vitamers, and preferably that the sampling is representative of the region the data are intended for. Furthermore, the absent of fat content has been an acceptance criterion, in order to make the most appropriate comparison between studies. Even though in fish, no correlation between fat and vitamin D in fish species has been observed [62], fat-soluble vitamins have been reported to be associated with content of fat in pork, milk, and beef [71,72,80].

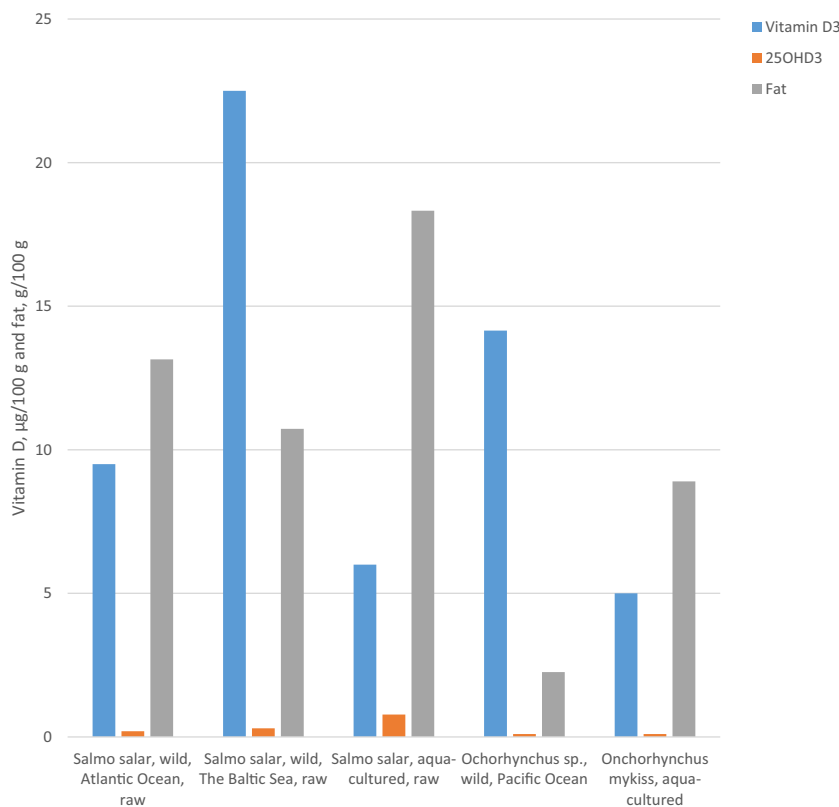
Cod liver oil is marketed as a supplement to diet e.g., with a content of approx. 200 µg vitamin D<sub>3</sub>/100 mL [13], which is far lower than reported for unprocessed

products [9,13]. The reports for vitamin D collected for food databases in aqua-cultured salmonids, *Salmon salar*, state content of 5.9 (2.3–8.2) µg vitamin D<sub>3</sub>/100 g, and for *O. Mykiss* 7.0 µg vitamin D<sub>3</sub>/100g [81–83]. These levels are lower than reported above for wild-caught *Salmo salar*. See Fig. 55.5. Though feeding trials in *Salmo salar* have shown an effect on the content of vitamin D in flesh by increasing vitamin D in the feed, the enhancement differs [63,84]. One study showed an increase to 9.5 µg vitamin D<sub>3</sub>/100 g using approx. 1500 µg vitamin D<sub>3</sub>/kg feed, while the other obtained content of 35 µg vitamin D<sub>3</sub>/100 g by feeding 3500 µg vitamin D<sub>3</sub>/kg feed. It is hypothesized that the effect of feeding could be strain-specific, which has been observed for salmon in a growth experiment [63,85]. A similar feeding experiment in *O. mykiss* showed no effect on vitamin D in the flesh of increased vitamin D in the feed [86]. In 2019 in the EU, the content of vitamin D in fish feed was regulated, and for salmonids increased from a maximum level of 75 µg/kg feed to 1500 µg/kg feed [87]. The effect of this on salmon vitamin D levels will be followed in future studies, but by 2022, vitamin D in salmon in the Danish market had not increased (Jakobsen, personal communication). Another aqua-cultured species is Tilapia, which is reported to contain 20–40 µg vitamin D<sub>3</sub>/100 g [88,89], while the seawater aqua-cultured species Giant sea perch and Short-bodied mackerel contained 3 µg/100 g [89]. The content of vitamin D<sub>3</sub> is included, as the other vitamers are insignificant in wild-caught fish, though aqua-cultured salmon contains 25(OH)D<sub>3</sub> in an amount of approx. 10%–20% of vitamin D<sub>3</sub> [90].

The capability of fish to synthesize vitamin D on exposure to ultraviolet UVB-light (300 nm) was examined in the freshwater fish tilapia (*Tilapia mossambica*) and resulted in a significant increase of vitamin D<sub>3</sub>. The content of vitamin D increases from 4 µg/100 g to 20 µg/100 g when the fish was exposed to UVB-light [91]. The UVB-light at 300 nm can travel through to a depth of 20 m in the sea [92]. Thus, UVB-light does travel through water, and especially the fish species living in the limnetic zone, including swordfish, tilapia, and silver barb, may gain vitamin D from this route. Otherwise, it is hypothesized that the vitamin D in consumed plant- and zooplankton is the main source of vitamin D for fish [69]. Regarding exposure to blue light on the skin of trout (*O. mykiss*), one group reported production of vitamin D by using blue light 380–480 nm, while another group did not see any vitamin D production by using light at 410–500 nm on the skin of Atlantic salmon (*S. salar*) [93,94].

In general, eggs (cage or free-range) are a good source of vitamin D. The reported result from three food data bases are in average between 0.6 and 2.5 µg vitamin D<sub>3</sub>/100 g and 0.13–1.0 µg 25(OH)D<sub>3</sub>/100 g [73,95,96].





**FIGURE 55.5** Content of vitamin D and fat in raw, salmonids—wild caught and aqua-cultured [58,63,65,81,145].

For these values, no information on the vitamin D content in the feed is available. A linear dependence of vitamin D in egg to vitamin D in the feed up to a maximum 620 µg/kg feed, provide the possibility to produce eggs that contain 61 µg vitamin D<sub>3</sub>/100 g without harmful effect for either hens or egg strength [97]. The possibility to manipulate the content of vitamin D in eggs has been shown through feeding and by UVB-exposure [2,60,97–99]. The maximum approved level of vitamin D<sub>3</sub> in the EU is 80 µg/kg feed, which is estimated to give 2.1 µg vitamin D<sub>3</sub>/100 g egg and 0.5 µg 25(OH)D<sub>3</sub>/100 g egg. Another possibility is to use 25(OH)D<sub>3</sub> as vitamin D source in the feed, which will increase the content of 25(OH)D<sub>3</sub> in the egg, but not in a linear relationship, and result in a minor increase of vitamin D<sub>3</sub> in the egg [97]. UVB-light in chickens facilities could, within 6 weeks of exposure of the legs of the hens, increase vitamin D content fourfold, which approximately resulted in a content in the eggs of 6.3 µg vitamin D<sub>3</sub>/100 g and 0.9 µg 25(OH)D<sub>3</sub>/100 g [100]. An alternative biofortification strategy is UVB-exposure of egg yolk, which also shows an increase in the content of vitamin D<sub>3</sub> due to the presence of 7-DHC [97].

Studies conducted for food databases show content of vitamin D in the milk of 0.26 µg vitamin D<sub>3</sub>/100 g milk

fat [73]. If an 85% retention during pasteurization is assumed (see below), the content is estimated to 53% of the “natural” level (see above). When feed containing the recommended level of approx. 7 µg vitamin D/kg feed (assumption 70 kg feed/day) was utilized, and no omission of a winter decline of vitamin D in conventionally produced milk was observed [71]. In the EU the approved max. is 100 µg vitamin D/kg feed [101], which could enhance the content in the milk during winter-time. Cows are very efficient in producing vitamin D in their skin by sun-exposure, and sun-exposure will cause the production of vitamin D in all parts of the cows [102]. Thus an alternative is the use of UVB-exposure in indoor dairies, which has shown an increase of vitamin D to “natural” levels in milk [103]. Furthermore, the applicability of UVB-treatment of milk was used in the 1940’s to enhance the content of vitamin D in milk, and the process is approved in the EU [104].

### 5.2.1 Pork

In Denmark, the levels of vitamin D in conventional pork (4.2% fat) are, on average, 0.052 µg vitamin D<sub>3</sub>/100 g and 0.050 µg 25(OH)D<sub>3</sub>/100 g [105], while reported content in lean meat of cuts from Canada is 0.14 µg vitamin D<sub>3</sub>/100 g and 0.09 µg 25(OH)D<sub>3</sub>/100 g [88]. These values indicate that farmers in the two

countries may use different levels of vitamin D in pig feed. Pigs in conventional farming are raised indoors, and thus the level of vitamin D in pork products depends on the content of vitamin D in the feed and/or the source, which can be either vitamin D<sub>3</sub>, 25(OH)D<sub>3</sub>, or vitamin D<sub>2</sub> [106,107]. Trials in pigs fed with the maximum allowed level (in the EU at 50 µg/kg feed) of vitamin D<sub>3</sub> showed a vitamin D content in pork (2.2%–2.1% fat) at max. 0.14 µg vitamin D<sub>3</sub>/100 g and 0.19 25(OH)D<sub>3</sub>/100 g [106,107]. In research facilities, UVB-exposure has been used in indoor stables for pigs, which by exposure for 4 weeks achieve content in pork (1.5% fat) at 0.37 µg vitamin D<sub>3</sub> and 0.24 µg 25(OH)D<sub>3</sub> per 100 g [108]. These contents are higher than by feeding, and minced pork (10% fat) could provide the consumer in 100 g with 1.7 µg vitamin D<sub>3</sub> and 0.3 µg 25(OH)D<sub>3</sub>. The content in the rind of UVB-exposed pigs was even higher at 31.6 µg vitamin D<sub>3</sub>/100 g.

Similar to the enhancement of the content of vitamin D in egg-yolk and milk, UVB-exposure to pork rind may enhance the content of vitamin D. The content of 7DHC in processed pork rind (pork crackling), can be used to produce cracklings with tailored content of vitamin D e.g., 10 or 20 µg/100 g by UVB-exposure [109]. Interestingly drinking water with ultrahigh content of vitamin D<sub>3</sub> (2.8 mg/100 mL) during lairage time prior to slaughter improved the tenderness of the meat [110].

### 5.2.2 Chicken

Food composition data for minced chicken (7.5% fat) revealed 0.10 µg vitamin D<sub>3</sub>/100 g and 0.44 µg 25(OH)D<sub>3</sub>/100 g, while data from Finland (9.7% fat) showed 0.29 µg vitamin D<sub>3</sub>/100 g and 0.25 µg 25(OH)D<sub>3</sub>/100 g, and for a French chicken (4.4% fat) 0.45 µg vitamin D<sub>3</sub>/100 g and 0.29 µg 25(OH)D<sub>3</sub>/100 g [62,73]. Thus, chicken seems to show variations in the distribution of vitamin D<sub>3</sub>/25(OH)D<sub>3</sub> due to different vitamin D sources in feed, as both vitamin D<sub>3</sub> as well as 25(OH)D<sub>3</sub> are approved in feed for chickens [111]. UVB-exposure to hen is most effective to the nonfeathered areas such as legs, comb, and wattles and may show an increase per 100 g of approx. 0.3 µg vitamin D<sub>3</sub> and 0.15 µg 25(OH)D<sub>3</sub> [60].

### 5.2.3 Beef

The first data on vitamin D in beef (average 6.1% fat) originated from Finland, which identified 0.06 µg vitamin D<sub>3</sub>/100 g and 0.06 µg 25(OH)D<sub>3</sub>/100 g [14], while Norwegian minced meat (13.2% fat) shows 0.09 µg vitamin D<sub>3</sub>/100 g and 0.003 µg 25(OH)D<sub>3</sub>/100 g [112]. The most comprehensive study of vitamin D in raw beef cuts included 48 individual samples of six difference cuts bought on the Danish market. The samples were of Danish origin and imported beef from 10

countries in Europe and South America. On average 9.5% fat, 0.093 µg vitamin D<sub>3</sub>/100 g, and 0.14 µg 25(OH)D<sub>3</sub>/100 g [73]. A dependence of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> of fat content was not observed, while such dependence was identified in cuts from the same animal [72,73]. Investigation of the differences between the three breeds of steers, showed a significant difference in the content of vitamin D<sub>3</sub> in strip loin, while no differences were observed in the liver [113]. The content of vitamin D in beef may be manipulated to a higher content by increasing the level of vitamin D in the feed. The approved sources of vitamin D<sub>2</sub> are less effective than the sources of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> to transfer vitamin D to animal flesh [114,115]. Furthermore, the effect of higher levels of vitamin D in feed or extra vitamin D prior to slaughter increased tenderness of the flesh [114,116].

### 5.2.4 Organs

The liver in fish is a storage site for vitamin D. In humans, as a representative of mammals, an estimate is that 65% of vitamin D in the body is represented by vitamin D, and 35% as 25(OH)D<sub>3</sub>, and that vitamin D is primarily stored in fat, while 25(OH)D is distributed throughout in fat, serum, muscle, and other tissues [117]. Few specific data are available for vitamin D vitamers in the liver, kidney, and heart in pigs, beef, and lamb. The highest level is reported in the liver, though the reported content shows huge differences in the range 0.05–1.2 µg vitamin D<sub>3</sub>/100 g and 0.08–0.53 µg 25(OH)D<sub>3</sub>/100 g [14,73,118]. See Fig. 55.6.

### 5.2.5 Mushrooms

UVB-exposure during or postharvest of mushrooms, such as white button (*Agaricus bisporus*) resulted in an enhanced content of vitamin D<sub>2</sub> [76,119,120]. The UVB-exposure to mushrooms produces vitamin D<sub>2</sub> from lumesterol and tachysterol, but the amount depends on temperature due to the equilibrium between previtamin D ↔ vitamin D [121,122]. Mushrooms with vitamin D content are marketed, and in the EU the process is approved for a content of vitamin D up to 20 µg vitamin D/100 g [123] and in mushroom powder as an ingredient up to 2.1 µg vitamin D<sub>2</sub>/100 g in the final product [124,125].

### 5.2.6 Bread and other alternative vitamin D products

UVB-treatment of yeast has been known since the 1930s and is approved in the EU as a novel food to be used in bread production in an amount of 5 µg vitamin D<sub>2</sub>/100 g [126,127]. Vitamin D content has been reported in avocado, but recent investigation by the Danish Food Database showed a content <0.1 µg/100 g (vitamin D<sub>3</sub> and vitamin D<sub>2</sub>) [128,129]. Research into the effect of

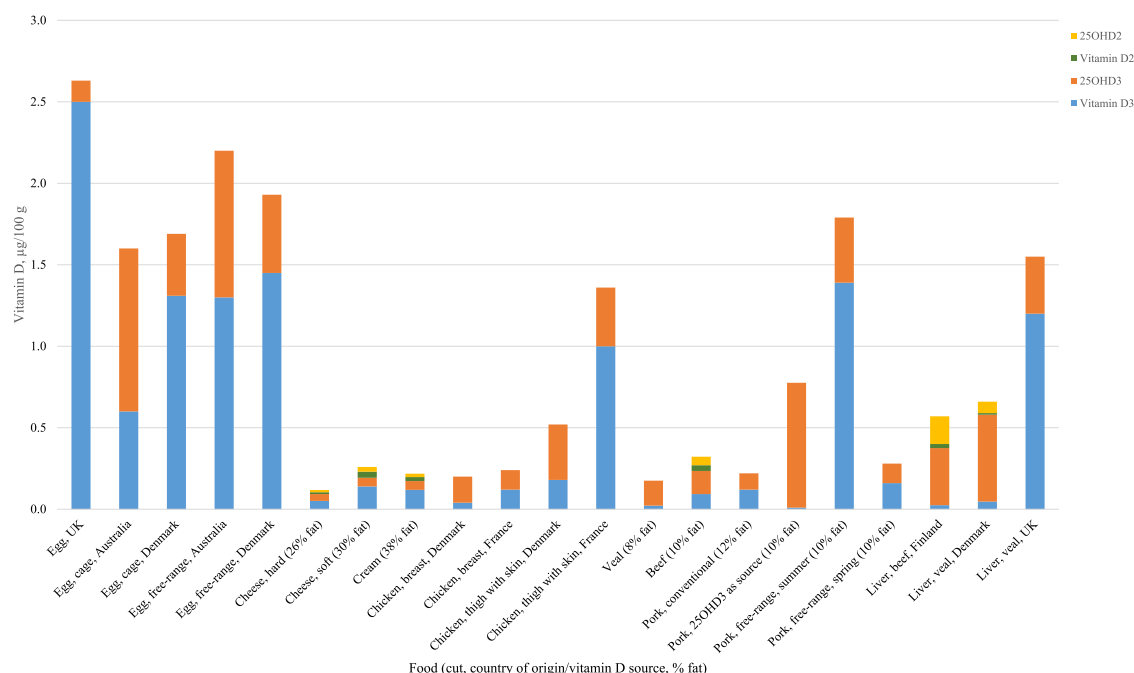


FIGURE 55.6 The content of vitamin D in a variety of raw, food [14,73,81,95,118,167].

UVB-exposure to vegetable oil (avocado, canola, and wheat germ) shows that especially wheat germ oil may achieve a content of 8 µg vitamin D<sub>3</sub>/100 g and of 150 µg vitamin D<sub>2</sub>/100g [130].

For insects in a research feeding study, super worms are reported to contain 1.3 µg vitamin D<sub>2</sub>/100 g [131], while UVB exposure of yellow worms showed an increase to 15 µg vitamin D<sub>3</sub>/100g [132]. Furthermore, recent research indicates the possibility of genetically producing vitamin D in tomato [133].

### 5.3 Fortified food

A recent survey of fortification policies in European countries revealed that only two countries (Sweden and Belgium) like Canada in the North American continent have mandatory fortification of margarine and spreadable fat (7–28 µg vitamin D<sub>3</sub>/100 g), and 1 µg vitamin D<sub>3</sub>/100 g in Sweden (milk and sour milk) and in Canada (milk) [134–137]. Other vehicles are used in the voluntary fortification of food products. For example, in Belgium, cereals and cakes, juice, and lemonade may be fortified. More information on vitamin D fortification in food is presented in Chapter 58.

Vitamin D used in fortified food usually derives from ergosterol in yeast (vitamin D<sub>2</sub>) or lanosterol from wool (vitamin D<sub>3</sub>) [31]. The challenge for the producer is the stability of the added vitamin D due to the possible degradation mentioned above. Different encapsulation strategies have been studied, including different milk protein emulsifiers, vitamin D binding to milk protein,

vitamin D encapsulated by soy protein isolates, and vitamin D incorporated in mixed micelles have been tested to improve the solubility and stability toward heat and light [138–141].

### 5.4 Processing of food—effects on degradation of vitamin D

This section will provide information on how storage, household cooking, and industrial processes can affect vitamin D in foods. The combination of food and processing procedures is numerous but reported results on the changes in vitamin D during such processing are scarce. Some food, such as fruit, is eaten raw while others, such as meat and eggs, are processed. The reason for this is to improve edibility, digestibility, and palatability, but it might also improve nutritional value and decrease the risk to cause illness (due to microorganism). Especially for the industry, it is essential to enhance shelf life. Food processing can also give rise to new compounds (toxic or nutritional relevant) and decrease the palatability as well as the nutritional value.

Vitamin D as pure crystals or in solution is sensitive to light, heat, pH, and oxidation. The changes in the content of vitamin D due to the processing of food may be caused by degradation, and these will be evaluated, and described in detail in sections below: light (UVB/visible); heat (pasteurization/Boiling/Baking/Microwave); pH; Oxidation (oxygen/singlet oxygen); moisture content; and others processes such as drying/canning/fermentation. In the following

sections, retention of vitamin D is provided either as true retention (TR) or apparent retention (AR) and calculated on data for DM in the original paper. Content is given in per 100 g of edible portion, and e.g., for egg yolk transfer from per g DM to per 100 g fresh weight based on 7 g DM per egg yolk, 33% of an egg is yolk [97,142].

#### 5.4.1 Effect of exposure to light—not UVB

Vitamin D-fortified milk exposed to light showed a decrease in the content of vitamin D [29], which was later attributed to the content of riboflavin and the ability of riboflavin to act as a photosensitizer (see above). Thus, light should be omitted during the processing of milk to protect degradation of vitamin D. However, another study also revealed a loss of vitamin D if stored in darkness at 4°C for 21 days, which resulted in a retention in the range of 77%–88% [143].

A soybean oil fortified with vitamin D<sub>3</sub>, was stored in the dark for 1 month at 30°C followed by sunlight exposure for 1 month at room temperature, which should imitate the storage condition from manufacturer to consumer. The retention was 32%–39%, and the results indicated that  $\alpha$ -tocopherol as an antioxidant had a protective role in the degradation of vitamin D [144].

#### 5.4.2 Effect of temperature

Storage of canned swordfish in different filling media (olive oil, corn oil, sunflower oil, and high oleic sunflower oil) for 12 months at room temperature in darkness showed a vitamin D decrease of approximately 14%, which was independent of the filling oil [145]. Storage of eggs at room temperature followed by 1 week at 6°C showed that the natural content of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> in eggs, had an AR >93% (Mattila, 1999). The degradation of vitamin D<sub>2</sub> in biofortified mushroom powder showed a first-order kinetic degradation at 20 degrees to 40°C, and the degradation rate increased by higher water activity [146]. Mushrooms stored at –20°C for 9 months showed no decrease [147], and storage at –18 to –20°C of homogenized samples of e.g., salmon, meat, and eggs, in dark and oxygen free for at least 10 years show no change in the content of vitamin D (Jakobsen, personal communication).

Vitamin D<sub>3</sub> in soybean oil stored for 1 month in the dark at 30°C showed ~100% retention [144]. Vitamin D-fortified dairy products such as milk, yoghurt, and cheese stored at 4°C have shown no loss within the shelf life of the product, neither was observed any loss in the content of vitamin D in cheese during ripening for up to 9 months [148–150]. Others stored vitamin D fortified Cheddar cheese for 7 months and report retention of 60%–89% [151]. Vitamin D-fortified milk powder stored at 20°C and 40°C, also showed a significant loss with a retention of 76%–62% after 12 months [152].

Vitamin D<sub>3</sub> fortified flour stored at 25°C or 45°C combined with relative humidity of 33% degraded over 120 days. The loss increased with the rise in temperature from 42% to 65% [153].

#### 5.4.3 Storage oxygenation

Vitamin D<sub>3</sub>-fortified skim milk treated with air showed a loss of vitamin D<sub>3</sub> of approx. 10% after 12 days [29].

#### 5.4.4 Relative humidity

Vitamin D<sub>3</sub> in flour stored at 25°C combined with an increase in relative humidity from 33% to 93% showed increased degradation after 120 days from 42% to 50% [153].

#### 5.4.5 Processing treatments

Industrial processing of swordfish (*Xiphias gladius*) included frying in olive oil (120°C for 6 min), which resulted in an AR of 84%. The fried swordfish was put in jars with different filling media (olive oil, corn oil, sunflower oil, and high oleic sunflower oil) and thermally treated at 115°C for 6 min. The sterilization caused no loss of vitamin D [145]. Boiling of trout (172°C–200°C for 20 min) showed a TR of 92% (78%–104%) [147]. Trout processed by eight different household cooking incl. boiling (110°C–210°C for 30–10 min), panfrying, steam cooking, and baking in an oven with and without foil showed an average retention of 96% (85%–114%). Only the lowest retention (85%) found for pan frying was significantly different from 100%, [154]. Cooking of nine different fish species (freshwater and marine) following household cooking in Thailand such as boiling (100°C for 4–10; one for 240 min), frying (110–150°C for 5–10 min), and grilling (230–250°C for 30–45 min) showed an average TR of 83% (47%–100%) (1 TR of 22% was evaluated as an outlier. Seven out of the 27 treatments had a TR significantly different from 100% [89].

In three independent studies, an egg boiled for 8–10 min showed vitamin D<sub>3</sub> a TR between 87% and 96% [95,155,156]. Processing of scrambled eggs (pan-fried for 3 min) and oven-baked (160°C for 40 min) retained 82% and 39% of vitamin D<sub>3</sub>. TR of 25(OH)D<sub>3</sub> showed similar levels as for vitamin D<sub>3</sub>.

For beef, the AR after traditional boiling, roasting, and braising of beef cuts, was 65%–69% [157,158]. Pork roasted with rind (in the oven 250°C for 20 min, 150°C until meat core 80°C) showed overall an AR of 85%, which for lean meat and lard were 100%, and for rind (crackling) 63% [80]. In contrast, crackling made from free-range pigs and UVB-exposed pork skin followed by baking (100°C for 60 min and 180 degrees for 45 min) showed a loss of 99 ± 1% [109].



Industrial processing by spray-drying vitamin D<sub>3</sub>-fortified milk resulted in an insignificant loss of vitamin D<sub>3</sub> [159]. In contrast, pasteurization of human donor milk in a water bath at 62.5°C for 30 min, resulted in a TR of vitamin D (vitamin D<sub>3</sub>, vitamin D<sub>2</sub>, 25(OH)D<sub>3</sub>, 25(OH)D<sub>2</sub>) of 80%–90% (assuming no change in weight) [160]. The temperature is below the risk for thermal isomerization to pyro/isopyrocalciferol, which was not the case in a study of cheese heated in the oven (232°C for 5 min), which caused an AR at 77%–80% (assuming no change in weight) [150].

Pan frying (5 min) of mushrooms resulted in a TR at 90% [147], while a variety of five processing methods (pan frying at high/low heat for 5min/20 min; boiling, in oven 70°C/90 min or 200°C/10 min) resulted in a TR of 74% (62%–88%) [154].

A model system for fortified canola oil (560 µg vitamin D<sub>3</sub>/100 mL) showed a TR of 100%, 68%, and 33%, at the temperatures of 100°C, 150°C and 180°C, respectively [161]. Heating of commercially fortified vitamin D<sub>3</sub> margarine in the oven (166°C for 40 min) and in a pan (3 min), showed a TR of 45% and 82%, respectively [155]. While sunflower oil heated in the oven (110°C for 30 min, 210 min, or 10 min), in a pan, or in a microwave for 1 min, resulting in TR for vitamin D<sub>3</sub> at 85%, 76%, 70%, and 84%, respectively [154]. Heating of vitamin D<sub>3</sub>-fortified oils (corn, sunflower, and canola) showed no effect for the type of oil used [162].

During the raising of dough fortified with vitamin D<sub>2</sub> and D<sub>3</sub> at 30°C for 60–120 min at room temperature no loss of vitamin D was observed [153]. Rye and wheat bread baked with vitamin D<sub>3</sub> fortified flour or vitamin D<sub>2</sub> enriched yeast, showed a TR in rye bread of 69% and 73%, respectively, while in wheat bread the TR was 85% and 89%. The cooking time for rye bread was 186°C for 60 min, which showed 90°C in the core of the bread, while for wheat bread the oven temperature was 170°C for 30 min, and the core temperature in the bread was 95°C. In contrast vitamin D<sub>3</sub> wheat bread baked at 200–205°C for 25 min showed a TR of 40%, and bread fried i.e., on a pan (170–180°C) for 10–20 s showed a TR of 10%, while chappatis fried on a pan (200–220°C) for 80–90 s resulted in a TR of 29% [153]. A cake baked at 172°C for 60 min to a core temperature of 100°C showed a vitamin D<sub>3</sub> retention at 39% [155], while cakes baked at 175–180°C for 25 min, resulted in a TR of 84%, and cookies baked at 200–205°C for 12 min the TR was 65% [153].

#### 5.4.6 Cooking—antioxidant/pH

In the boiling water of trout and mushrooms lemon juice was added as an antioxidant, but also lowered the pH (3.5–4.0). The TRs were 87% and 62% with antioxidants and 102% and 80%, respectively, without.

The authors stated no significant difference, thus further studies are needed to evaluate the possible effect [154].

#### 5.4.7 Food processing summary

Table 55.2 sums-up the treatment of foods which can cause vitamin D to become unstable or stable. Based on primary data collected for studies of household cooking a table showing the retentions is constructed. Only studies using specific chemical methods and reporting a minimum of two repetition for a cooking procedure are included. See Table 55.3. Retention studies need to be performed with strict guidelines such as timing, amount of food, temperature, and number of replicates (minimum three replicates). Furthermore, quality control similar to that performed for other analytical methods should be included. As mentioned, the optimal procedure is the use of true retention, which due to possible uncertainty should not exceed 120%.

Frying on a pan seems to degrade vitamin D to a higher extent than boiling, which is caused by the thermal degradation of vitamin D, potentially in combination with oxidation.

The significant difference between TR in rye bread and wheat bread seems not to be caused by the temperature but might be due to the higher content of water in rye bread. DM in rye bread is 54% and in wheat bread 64% [15]. The possibility of creating a model system that will illustrate the breakdown of vitamin D in foods is hampered by the challenges to determine each of the factors causing the degradation of vitamin D e.g., light, temperature, relative humidity/water activity, oxygen, and pH.

In the future, it would be beneficial to understand the degradation of vitamin D during the different treatments, and how to prevent this. High heat is obviously a cause for low retention, but it seems that water activity, relative humidity, and oxidation could be reasons for the relatively low retention during baking in the oven when the core temperature does not reach more than 100°C. Food databases may prioritize the content of raw food, and then the user will need to correct for retentions lower than 100% as shown in Table 3. Alternatively, food databases may prioritize to analyze processed food using a procedure, which is defined as the most common for the area/country of concern (UK; AUS). In this case, it would be beneficial to make the TR visible for the cooking process used.

### 5.5 Distribution of vitamin D vitamers in food and calculation of total vitamin D activity

The contribution of 25(OH)D<sub>3</sub> to vitamin D activity in food depends on the type of food, and not surprisingly,



**TABLE 55.3** Overall retention factors for vitamin D<sub>3</sub> in different foods by usual household cooking.

Type of food	Processing	Temp., °C	Time, min	Number <sup>a</sup>	% TR or % AR		Comments	References
					Average	Range		
Fish	Oven, pan-fried, boiled, microwave	100–250	4–45	40	86	49–114	AR estimated [145,166]	[89,145,147,154,166]
	Microwave	-	2–13	2	97	92–101	AR estimated for [166]	[154,166]
	Pan-fried	-	6–15	11	82	51–97	AR estimated [145,166]	[145,154,166]
Eggs	Boiled	100	10	2	92			[147,155]
	Scrambled on pan	-	3	1	82			[155]
	Oven	150	40	1	39			[155]
Mushroom	Oven, boiled, pan-fried	70–200	90–5	7	79	60–99		[154]
	Pan-fried	-	5	4	87	81–99		[147,154]
Meat (beef, pork)	Boiled, oven, fried	150–250	15–60	4	71	65–85	AR estimated [80,157]	[80,157,158]
Rind	Oven	100 + 180	60 + 45	1	1		Cracklings	[109]
Bread/cakes	Rye bread	186	60	1	69		Vitamin D2: TR = 73%	[155]
	Wheat bread	170–205	25–60	4	70	40–80		[153,155]
	Flatbread-panfried	-	10–90 s	2	19	10–29		[153]
	Cakes	172–205	12–60	6	74	64–85		[153,155]
Oil	Oven, fried, microwave	105–210	60–10	8	80	70–85		[154,162]
Margarine	Oven	166	40	1	45			[154,155]
Dairy prod	Human milk, pasteurized	62.5	30	16 <sup>b</sup>	85	80–90	Loss of 10%–20%	[160]
	Cheese, oven	232	6	1	79	77–80	Assume no weight changes	[150]

<sup>a</sup>Number of values, which each is based on two-four repetition.<sup>b</sup>16 individual samples.

**TABLE 55.4** Variation (SD,%) for individual samples analyzed for vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub>.

Food	Number of	Vitamin D <sub>3</sub>	25(OH)D <sub>3</sub>	References
	Samples	SD, %	SD, %	
Salmon, <i>Salmo salar</i>	10	23	39	[81]
Eggs, free range	5	76	34	[95]
Eggs, free range	12	31	21	[73]
Eggs, cage	5	14	29	[95]
Eggs, cage	14	34	24	[73]
Pork, free range, lean meat, summer	20	28	32	Do.
Pork, free range, lean meat, spring	20	64	27	Do.
Beef, topside	6	114	68	Do.
Beef, heart of rump	6	35	32	Do.
Beef, knuckle	6	76	53	Do.
Beef, brisket, point end	6	89	37	Do.
Beef, brisket, boneless	6	114	100	Do.
Beef, ribeye/entrecote	6	60	46	Do.
Beef, short loin	6	103	47	Do.

depends on the type of animal feed and access to sun and artificial UVB-exposure. In a country without mandatory fortification, and limited use of voluntary fortification, the amount of 25(OH)D<sub>3</sub> is approximately 20% and 33% of the ingested vitamin D<sub>3</sub> in adults and children, respectively [73]. The share decreases to approx. 11% with extra feeding of vitamin D<sub>3</sub> and/or use of UVB-exposure, but increase to approx. 67% (adult) and 108% (children) if 25(OH)D<sub>3</sub> is used as vitamin D source for animal husbandry [73].

It could be an easy task to calculate the total vitamin D activity in food by summing up the individual content of vitamin D<sub>3</sub>, 25(OH)D<sub>3</sub>, vitamin D<sub>2</sub>, and 25(OH)D<sub>2</sub>. Though debated, there is a consensus that vitamin D<sub>3</sub> and vitamin D<sub>2</sub> have the same bioactivity. Due to the minor difference in molar mass (vitamin D<sub>3</sub> ~ 384,64 and vitamin D<sub>2</sub> ~ 396,65) the content of vitamin D<sub>2</sub> is usually not corrected to vitamin D<sub>3</sub> equivalent. In contrast, there is no consensus for the activity of 25(OH)D<sub>3</sub> (or 25(OH)D<sub>2</sub>) compared to vitamin D<sub>3</sub>. The factor of five was introduced in research literature and then in food databases in the 1990's, but reviews have discussed the lacking documentation [56,163–165]. Due to the relatively high amount of 25(OH)D<sub>3</sub> compared to vitamin D<sub>3</sub>, it is essential to finally agree if, and if relevant, to what extent 25(OH)D<sub>3</sub> differs from vitamin D<sub>3</sub> in vitamin D activity.

The huge impact of vitamin D in food due to different animal breeds and fish strain, ocean versus farm living,

feeding system used, including access to sun- or artificial UVB-exposure, makes it essential to use area/country-specific food data. Food databases are essential, but it should be mentioned, that the number of original, primary, analytical data on food are very limited. Content for vitamin D in individual samples collected for food database and analyzed at the same laboratory show that the standard deviation for the average value for fish, eggs, pork, and beef for vitamin D<sub>3</sub> is in the range of 23%–114% (mean 62%; n = 14) and for 25(OH)D<sub>3</sub> in the range 27%–100% (mean 43%; n = 14). See Table 55.4.

The analytical precision for laboratories, which in general is <10%, (see above) does contribute to variation within a food type but is a minor contributor. Often composite samples are chosen to reduce analytical cost, thus no information on variation is available. Thus, it is essential that sampling is representative of the market intended. The limited number of sample types that have been analyzed forces data compilers to make assumptions, on which other food to choose as the most similar product, especially regarding the content of vitamin D. The data in food databases are advisable to use for the estimation of the dietary intake for a population. For an intervention study, the priority should be to analyze the food items, as the content of vitamin D depends on not only food type but the origin and type of animal feeding and breeding.

## 6. Conclusion

The lack of standardized methods, certified reference materials, and proficiency tests for quantification of 25(OH)D<sub>3</sub> in foods contribute to a higher uncertainty than for vitamin D<sub>3</sub>. Use of accredited methods should be used in projects aiming to establish new vitamin D data for food databases.

Vitamin D has a unique chemical structure, which enables isomerization to inactive forms due to light, heat, acid, oxygenation especially singlet oxygen, as well as water do have a negative influence on the stability in food. The content of vitamin D in food is very diverse—in wildlife, it depends on the season, breeding, and feeding.

Deficiency of vitamin D around the world is a problem, remembering the words from 1925, mentioned in the introduction, vitamin D may be increased through utilizing artificial UVB-light in indoor facilities and contribute to increased vitamin D levels in humans by biofortification making it possible to produce foods with targeted content of vitamin D.

## 7. Summary points

- The active vitamers in food are vitamin D<sub>3</sub>, 25(OH)D<sub>3</sub>, vitamin D<sub>2</sub> and 25(OH)D<sub>2</sub>.
- Vitamin D in food is susceptible to heat (>150°C) and singlet oxygenation.
- UVB exposure in husbandry or food enhance the content of vitamin D.
- Vitamin D in feed can enhance the content of vitamin D in food.
- Vitamin D in food is country or area specific.
- The total vitamin D activity of food might be estimated by summing up the content of the individual vitamers.

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## Further reading

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# Determinants of vitamin D levels from sun exposure: a global perspective

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## OBJECTIVES

- Understand the importance of sun exposure in determining 25-hydroxyvitamin D (25(OH)D) concentrations.
- Understand the seasonal nature of serum 25(OH)D concentrations.
- Learn about atmospheric effects on vitamin D production.
- Learn about endogenous effects on vitamin D production.
- Learn about behavioral effects on vitamin D production.

## 1. Introduction

Globally, serum 25-hydroxyvitamin D (25(OH)D) concentrations average about 50–55 nmol/L—nearly independent of latitude [1–6]. Although that similarity may seem paradoxical, the primary reason is that skin pigmentation varies inversely with latitude in general and that summer daylight times increase with latitude. The variation in pigmentation serves to balance production of vitamin D with protection against damage from free radicals and folate destruction by UV radiation [7]. Many observational studies report that adverse health outcomes are more likely as 25(OH)D concentrations decrease [8–11]. The Endocrine Society defines vitamin D deficiency as a 25(OH)D concentration below

50 nmol/L; vitamin D insufficiency is a 25(OH)D concentration of between 50 and 75 nmol/L [12]. Thus, about half the world's population is vitamin D deficient.

An estimate of the natural 25(OH)D concentration can be obtained from indigenous populations living in east Africa. A study examined two traditionally living populations there, one the pastoral Maasai and the other Hadzabe hunter-gatherers. Subjects had skin type VI, wear a moderate degree of clothing, and spend most of the day outdoors—but they avoid direct exposure to sunlight when possible. They had mean 25(OH)D concentrations of 115 nmol/L [13]. A study of pregnant women from five east African ethnic groups who consumed different amounts of fish found that solar ultraviolet B (UVB) exposure determined 25(OH)D concentrations and that the mean concentration was 115 nmol/L [14]. Those values correspond to an estimated daily production of vitamin D of 2000 IU [15]. On the other hand, a study in South Australia found that UV exposure led to a mean maximum 25(OH)D concentration of 89 nmol/L and was associated with an estimated mean weekly solar erythematous UV exposure of 1230 mJ/cm<sup>2</sup> [16]. Solar UV radiation at wavelengths 290–330 nm can destroy some vitamin D metabolites [17]. That effect limits the maximum 25(OH)D concentration resulting from solar UV exposure so that one cannot overdose on vitamin D from solar UVB exposure, as seen in the results from east Africa.

## 2. Vitamin D production from solar UVB exposure

An excellent systematic review and metaanalysis reported the impact of several factors on cutaneous vitamin D synthesis from UVB exposure [18]. One factor

is the part of the body exposed. The hands and face, which account for 3% of total body surface area (BSA), are eight times more effective in synthesizing vitamin D than the whole-body surface area. Another factor is that the increase in serum 25(OH)D concentration depends on baseline concentration. When the baseline is 21 nmol/L, the increase in terms of  $\Delta 25(\text{OH})\text{D}/\text{SED}/\% \text{BSA}$  is 0.093 where SED is standard erythemal dose, dropping to 0.004 for baseline 25(OH)D = 72 nmol/L.

Another review reported the change in 25(OH)D concentration with respect to simulated solar radiation [19]. It found that the change was linear in a logarithmic fashion: 10 standard vitamin D doses (SDDs), equivalent to about 12 SED, increased 25(OH)D by about 20 nmol/L, 100 SDDs by about 32 nmol/L, 500 SDDs by 46 nmol/L, and 1000 SDDs by about 52 nmol/L. A study in India found that men with a mean age of  $48 \pm 6$  years with 27% deficient and 41% insufficient 25(OH)D required greater than 1 h/day casual midday sunlight exposure to maintain 25(OH)D above 50 nmol/L and greater than 2 h/day for above 75 nmol/L [20].

### 3. Factors affecting 25-hydroxyvitamin D concentrations

Factors that affect 25(OH)D concentrations related to sun exposure fall under three broad categories [1]:

atmospheric and environmental determinants [2]; endogenous characteristics such as genetics and obesity; and [3] behavioral determinants. A recent paper reviewed the determinants of trends in vitamin D status [6]. Factors associated with decline include reduced sun exposure, increasing BMI, reduced consumption of vitamin D-containing foods, and the effects of urbanization, air pollution, less outdoor occupation, and poor socioeconomic status. Factors associated with increase include sun exposure on holidays, food fortification, increases in vitamin D supplementation, and increased physical activity.

#### 3.1 Seasonal changes in 25-hydroxyvitamin D

Solar UVB exposure is the major source of vitamin D for most people [21]. Table 56.1 shows winter and summer mean 25(OH)D concentrations for a representative sample of countries. The criteria for inclusion included that the 25(OH)D data were obtained after 2000, that they were for adults with data for elderly preferred, that the assays used were considered relatively accurate, and that a sufficient number of participants were included. The data from the various countries have some differences such as the age ranges of the participants, the selection of participants, and the periods considered summer and winter. Nonetheless, the data are very useful.

**TABLE 56.1** Seasonal changes in 25-hydroxyvitamin D (25(OH)D) concentrations.

Country, latitude	Population	Period	25(OH)D concentration (nmol/L) in:		References
			Winter	Summer	
Australia 19 to 43 degrees S	~250 in each of 4 cities, 18–75 years	2009–10	51 $\pm$ 23	74 $\pm$ 24	[22]
Australia 35 degrees S	3523		M 67 F 63	M 84 F 71	[23]
China 23 degrees N	M & F, <18 to >80 years	2018–19	73 $\pm$ 25	88 $\pm$ 25	[24]
Denmark 56 degrees N	F, 70–75 years	2002–03	49	67	[25]
Estonia 59 degrees N	367 M, F, mean 49 $\pm$ 12 years	2006	44 $\pm$ 15	59 $\pm$ 18	[26]
Germany 48.4 degrees N	1418 M + F, >65 years	2009–10	39 $\pm$ 3	63 $\pm$ 4	[27]
Great Britain 52 degrees N	M, 45 years F, 45 years	2002–04	34 36	74 71	[28]
Greece 39 degrees N	970 M & F	2010–12	48 $\pm$ 19	52 $\pm$ 20	[29]
Iceland 64 degrees N			56	74	[30]
India 29 degrees N	26,339 assays	2008–2017	52 $\pm$ 58	58 $\pm$ 56	[31]
Iran 38 degrees N	541 M & F, 5–60 years	2015	46 $\pm$ 24	55 $\pm$ 37	[32]

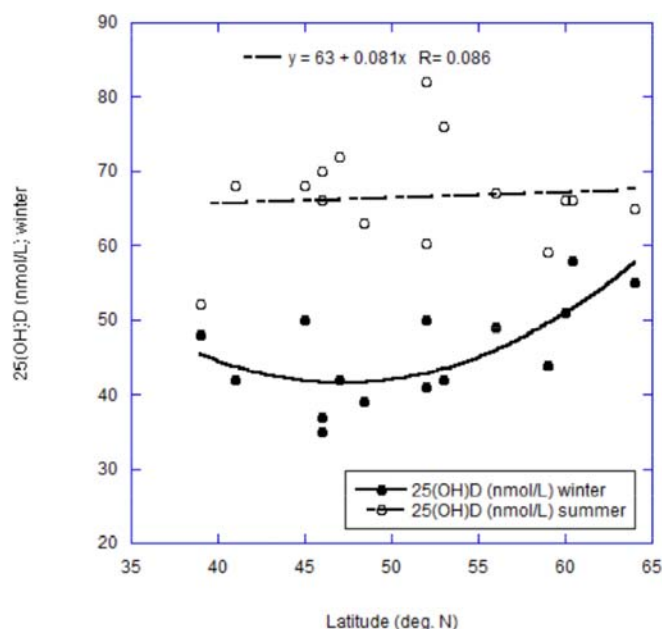
**TABLE 56.1** Seasonal changes in 25-hydroxyvitamin D (25(OH)D) concentrations.—cont'd

Country, latitude	Population	Period	25(OH)D concentration (nmol/L) in:		References
			Winter	Summer	
Ireland 53 degrees N	1132 adults	2008–10	42	72	[33]
Italy, 45 degrees N	2558 M, 8592 F, med age 62 years	2014	50 (IQR, 30–70)	68 (IQR, 47–89)	[34]
Japan 33 degrees N	312 M, 217 F, 21–67 years	2006	43 (Nov)	68 (July)	[35]
Japan 33 & 43 degrees N	107 M & F, 20–60 years	2018	37	53	[36]
Mongolia 48 degrees N	320 M, F, 20–58 years	2011, 2013	19 ± 8	56 ± 23	[37]
Netherlands 52 degrees N	201 M, 338 F	Ca 2010?	48	82	[38]
New Zealand 41 degrees S	2946, 18+ years	1996–97	40	75	[39]
Norway 60 degrees N		1999–2005	51	67	[40]
Portugal 41 degrees N	18–67 years	2015, 2016	42 ± 17	68 ± 22	[41]
Slovenia 46 degrees N	280 M&F	2017–18	35	77	[42]
Sweden 60 degrees N	M & F, 210 in summer, 58 in winter	2010–11	55 ± 18	66 ± 18	[43]
Switzerland 47 degrees N	1308 M + F	2010–11	42 ± 3	72 ± 3	[44]
Switzerland 46 degrees N	M&F, BMI = 25	2003–08	35	66	[45]
Turkey 40 degrees N	F, 21–52 years, office workers	2008	35	71	[46]
United Arab Emirates 24 degrees N	M & F, university students	2009–10	31	21	[47]
United States 26–48 degrees N	Adults	2007–09	54	71	[48]

Fig. 56.1 also raises the question of what determines serum 25(OH)D concentrations in winter in the absence of solar UVB. Two recent articles made the case that the primary mechanism for maintaining 25(OH)D concentrations in the absence of solar UVB is the storage of 25(OH)D in muscle cells [49,50]. They proposed that parathyroid hormone controlled the recirculation process. The linear regressions in Fig. 56.1 can also be used to estimate the relative contributions of oral vitamin D intake and solar UVB in winter. Let us assume that all of the oral intake of vitamin D is from food. Animal sources are the only source of vitamin D, ignoring that from fungi, with meat and fish accounting for the largest share [51]. Data from the Food and Agriculture Organization can be used to estimate the amount of animal products in the national diets (<https://www.fao.org/faostat/en/#data>, accessed June 15, 2022). Fig. 56.1 is a plot of summer and winter serum 25(OH)D concentrations for European countries using the

data from Table 56.1. What is interesting is that summertime mean concentrations for adults are near 68 nmol/L for all latitudes, while wintertime concentrations have a U-shaped relationship with latitude. The summertime effect can probably be explained as due to a combination of decreasing amount of skin pigmentation [7] and increased length of daily sunlight in summer with increasing latitude. The wintertime effect at high latitudes can be explained by the higher consumption of animal products that serve as sources of vitamin D at higher latitudes [51–53] as well as higher intake of vitamin D supplements [30] and food fortification such as in Finland [54]. The slight increase in 25\*(OH)D at lower latitudes can be explained by a longer duration of producing vitamin D from solar UVB exposure (see Ref. [55]). The finding regarding winter concentrations suggests that more countries should consider vitamin D fortification of food to raise wintertime 25(OH)D concentrations [56–58].





**FIGURE 56.1** Latitudinal dependence of summertime and wintertime 25(OH)D concentrations for adults in European countries using data in Table 56.1. The equation for the winter regression fit is  $25(\text{OH})\text{D} (\text{nmol/L}) = (170 - 5.4 \times \text{Latitude} + 0.057 \times \text{Latitude}^2) (\text{nmol/L})$ ,  $r = 0.72$ .

### 3.2 Geographic location and ultraviolet B doses: seasonal variations

The most important factor affecting vitamin D production from solar UVB exposure is the solar zenith angle (SZA). The spectral region for solar UVB reaching the earth's surface is 290–315 nm. Given the short wavelength, UVB is strongly scattered by atmospheric molecules. According to Rayleigh's model, scattering varies as the inverse fourth power of wavelength. In addition, stratospheric ozone affects UVB transmission. Thus, the longer the atmospheric path, the less UVB reaches earth's surface. A graph shows that UVB radiation at 310 nm reaching earth's surface drops by an order of magnitude in going from an SZA of 25–75 degrees [55]. For San Francisco on June 8, the SZA reaches 25 degrees at 1300 h (1:00 p.m.), falling to 75 degrees at 0745 and 1750 (<http://keisan.casio.com/exec/system/1224682277>). The general rule is that one can produce a reasonable amount of vitamin D for SZA <45 degrees. The SZA reaches these values that day at 0930 and 1610.

Figure 3 in Engelsen [55] indicates that vitamin D can be produced rapidly from solar UVB the entire year for latitudes <20 degrees, rising to 65 degrees in summer. However, it is impossible to produce vitamin D at the end of the year for latitudes >46 degrees, rising to 65 degrees by early March. From these calculations, it is estimated that for Fitzpatrick skin type III, one-quarter of a minimal erythemal dose over one-quarter of the body

would produce 1000 IU [59]. In Florida, that would take 8–15 min near solar noon, depending on the season.

The calculations in Engelsen [55] were based on the International Commission on Illumination (CIE) action spectrum for previtamin D production [60]. A recent paper pointed out some of the problems with the CIE action spectrum such as the use of a bandwidth of several nanometers in its determination [61]. Another was developed by the Dutch National Institute for Public Health and the Environment (RIVM) in the Netherlands [62]. In addition, that paper points out that prolonged UVB exposure saturates vitamin D production and that wavelengths between 310 and 330 nm cause photodecay of vitamin D metabolites. Although an improved action spectrum would probably make a few days change vitamin D production rates, the general conclusions reached using the CIE action spectrum offer good guidance for now.

Additional factors to consider include altitude, surface type, and aboveground features. UVB intensity increases about 19% per 1000 m for an SZA of 20 degrees [63]. The effect of altitude is evident in maps of vitamin D-producing UVB doses in the United States [64]. Different surfaces reflect different amounts of solar UVB. For overhead sun, reflectance varies from 10% for water, 12% for land, 23% for an alpine pasture, to 87% for new dry snow [63]. However, reflectance varies as a function of SZA; anyone who has spent much time on the water in summer knows how easy it is to get sunburned because of the high reflectance at higher SZAs. Living in forested regions reduces UVB doses. As a result, populations inhabiting forested tropical regions for centuries to millennia have lighter pigmentation than those who live in tropical plain regions [7].

### 3.3 Meteorological factors

Clouds can reduce the amount of UVB radiation reaching earth's surface. The degree of attenuation depends on the optical thickness/density of the cloud, the amount of cloud cover, and the SZA [65]. UVB can penetrate thin clouds. Coastal regions also can have considerable fog, which also attenuates UVB radiation reaching the surface. A good example is San Francisco, where the marine air passes through the Golden Gate in summer to cool the Sacramento Valley, bringing both clouds and fog.

Air pollution reduces vitamin D production by attenuating solar UVB radiation reaching earth's surface [66] as well as by reducing time spent outdoors [67]. In addition, since air pollution particulate matter perturbs over 500 genes including multiple proinflammatory

cytokines which vitamin D can counter [68], and since smoking reduces 25(OH)D concentrations [69], it is very likely that air pollution also reduces 25(OH)D concentrations due to vitamin D combating the inflammation and oxidative stress caused by the pollution [68]. The effect of urban pollution has been documented in studies in Belgium [70], China [71], France [72], India [73], and Iran [41,42]. Although aerosols may contribute most of the attenuation, gases that absorb in the UVB spectral region such as ozone and sulfur dioxide also can contribute [70]. In highly industrial regions of China and India, pollution can attenuate UV radiation by up to 50% [71]. Air pollution may also help explain the higher rates of vitamin D deficiency in urban regions than in rural regions.

### 3.4 Urban versus rural residence

Living in urban regions is often associated with lower 25(OH)D concentrations. A study of the effect of urbanization on South African women explored the reasons for lower 25(OH)D concentrations for urban dwellers. Several factors were investigated. The most important ones identified were higher urban rates of obesity, lower urban rates of physical activity levels, and greater urban alcohol consumption [74]. Another study from South Africa reported lower 25(OH)D concentrations among adolescents with alcohol use disorder [75]. Another reason for lower 25(OH)D concentrations in urban regions is due to a greater preponderance of indoor occupations [76]. High-rise buildings also reduce the UVB radiation reaching the surface [77]. A study of women of child-bearing age in Vietnam found slightly higher 25(OH)D concentrations in the Hai Dong province (85 nmol/L) than in Hanoi City (78 nmol/L) [78]. A study in Malaysia found that rural women spent much more time in the sun than urban women ( $\sim 8$  vs.  $\sim 3$  hour/day, respectively, resulting in higher 25(OH)D concentrations,  $\sim 70$  versus  $\sim 32$  nmol/L, respectively), even though urban women exposed more skin surface area than rural women [79]. A study of elderly Koreans found higher 25(OH)D concentrations in rural than in urban residence (66 vs. 43 nmol/L, respectively) [80].

### 3.5 Travel to sunny locations in winter

People who live at high latitudes and travel to sunny locations during winter have higher 25(OH)D concentrations. A study of Swedish women found that a winter sun vacation was associated with a 14.5-nmol/L increase in 25(OH)D concentration—greater than the 11.0-nmol/L increase associated with a daily intake of 300 IU of vitamin D from reduced fat dairy products [81]. A study in Europe found that sun holidays increased 25(OH)D

concentrations from 49 to 71 nmol/L for Danes and from 56 to 73 nmol/L for Spaniards; ski holidays increased 25(OH)D concentrations for Danes from 51 to 59 nmol/L [82]. However, the study also found significant increases in T–T dimers (also known as thymine–thymine or pyrimidine dimers), a biomarker of DNA damage, measured in the urine.

## 4. Endogenous characteristics

### 4.1 Effect of age

Vitamin D is produced in the skin when UVB acts on 7-dehydrocholesterol, followed by a thermal reaction [83]. As people age, the amount of 7-dehydrocholesterol in the skin changes. On the basis of those concentrations in the dermis and epidermis [84], the ability to produce vitamin D in the skin decreases linearly with time from 10 years of age, falling to about 50% lower ability by 70 years of age [83]. A study in Hungary found that August 25(OH)D concentrations decreased with age, from 42 nmol/L for those aged 0–9 years to 21 nmol/L for those aged 80–89 years [85]. This finding is probably due to a combination of younger people spending more time in the sun and producing vitamin D from solar UVB more efficiently. However, a study found that the US children do not generally spend enough time in the sun to meet nominal vitamin D requirements [86].

### 4.2 Obesity

The higher the body mass index (BMI), the lower the 25(OH)D concentration is likely to be [87]. One plausible explanation for this finding is vitamin D sequestration in adipose tissue [88]. Another is volumetric dilution [89]. A third is decreased hepatic 25-hydroxylase activity due to decreased expression of CYP2R1 [90]. Given the worldwide increases in rates of obesity, global 25(OH)D concentrations related to obesity will continue to decline.

### 4.3 Genetics

In a study of exposure to UVB lamps in winter in Denmark, 22 healthy participants achieved 25(OH)D concentrations from 85 to 216 nmol/L [91]. Baseline 25(OH)D concentrations accounted for 55% of the variance, whereas age, polymorphisms in the vitamin D receptor gene, height, and constitutive skin pigmentation accounted for 15% of the variance. Genes involved in the vitamin D metabolic pathway also can affect 25(OH)D concentrations. Alleles of these genes are associated with different 25(OH)D concentrations. The candidate genes are *DHCR7*, *CYP2R1*, and *GC* as shown by Brouwer-Brolsma [62]. *DHCR7* encodes the enzyme

7-dehydrocholesterol reductase. This enzyme catalyzes the conversion of 7-dehydrocholesterol into cholesterol in the skin, thus preventing that 7-dehydrocholesterol from being metabolized into previtamin D. *CYP2R1* encodes the liver enzyme that converts vitamin D to 25(OH)D. *GC* encodes the vitamin D-binding protein, which transports vitamin D metabolites to different organs, tissues, and cells. In a study involving 2857 Dutch men and women older than 65 years, 35% of 25(OH)D concentrations were explained by a model including sun exposure, oral vitamin D intake, and genetic factors [62]. In the model adjusted for age, BMI, years of education, smoking, alcohol consumption, physical activity, and self-reported health, the factors significantly affecting 25(OH)D concentration were the *GC* gene ( $P = 0.005$ ), being outside in the past 2 weeks ( $P = 0.01$ ), and sunlamp use ( $P = 0.03$ ). *CYP2R1* was marginally insignificant ( $P = 0.07$ ).

Another paper found that people with certain alleles of *CYP2R1* and *GC* had the smallest increases in 25(OH)D concentrations after UVB exposures and the largest decreases in 25(OH)D concentrations after 6 months of consumption of vitamin D<sub>3</sub>-fortified bread and milk [92]. Thus, genetics can modestly affect 25(OH)D concentrations from UVB exposure and oral vitamin D intake. Genome-wide association studies (GWAS) have identified genes associated with 25(OH)D concentration. A GWAS involving up to 79,366 participants of European descent added two loci harboring genome-wide significant variants of 25(OH)D concentration, rs8018720 in *SEC23A*, and rs10745742 in *AMDHD1* to four already known (*GC*, *NADSYN1/DHCR7*, *CYP2R1*, *CYP24A1*) [93]. Another GWAS involving 417,580 Europeans identified 143 independent loci [94].

## 5. Behavioral determinants

### 5.1 Use of sunscreen

Sunscreens generally block erythral wavelengths (<325 nm) well. The thicker the layer of sunscreen applied, the less the UVB reaches the skin [95]. Thus, if not applied thickly enough, some UVB will reach the skin [96]. In addition, sunscreen wears off. People who habitually apply sunscreen when in the sun will have lower 25(OH)D concentrations than those who do not, assuming similar sun exposures [97]. Many women's cosmetics contain sunscreen (<http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=700.35>). The primary reason for putting sunscreen in cosmetics is probably to reduce the risk of elastosis (wrinkling) [98].

An alternative to the use of sunscreen to reduce risk of erythema and DNA damage is to raise serum

25(OH)D concentrations using vitamin D<sub>3</sub> supplements. An intervention study conducted in Cleveland, OH, United States, found that raising 25(OH)D concentration to above 100 nmol/L using 200,000 IU of vitamin D<sub>3</sub> resulted in very little erythema for a sunburning UV dose compared with those supplemented with 100,000 IU vitamin D and achieving 25(OH)D near 75 nmol/L [99]. The mechanism indicated was inhibition of inducible nitric oxide synthase and TNF- $\alpha$  by activated macrophages [100], thereby reducing risk of inflammation. The Cleveland group also suggested using high-dose vitamin D<sub>3</sub> to treat sunburn [101]. The work was endorsed in an editorial by Bikle [102].

### 5.2 Shade

Atmospheric molecules and aerosols strongly scatter UVB radiation. There are two types of scatter involved: Rayleigh scattering is involved for particles up to about a 10th of the wavelength of light, while Mie scattering occurs for larger particles. Atmospheric molecules have a diameter of 0.4 nm, which is much smaller than 300 nm. Rayleigh scattering varies as the inverse fourth power of wavelength, whereas aerosols scatter to a lower extent, depending on particle size. The effect of scattering explains why the clear sky is blue and clouds are white during midday and orange at sunrise and sunset. Thus, UV exposure is related to direct, diffuse, and reflected UV radiation. A model calculation for Payerne, Switzerland, estimated that direct UV erythral radiation contributed 15%–24% of annual exposure, whereas diffuse radiation explained about 80% of cumulative erythral dose [103]. A study reported that for SZA between 35 and 60 degrees, previtamin D production under trees and umbrellas was about half of that in full sun [104]. A later paper reported that in Australia, the best time to expose the body to UV radiation while using a shaded environment with a sky view of >40% was when the SZA was <45 degrees [105]. Using that approach would reduce total UV exposure by 37%–58%.

### 5.3 Messages to get UV exposure mornings and afternoons (the shadow rule)

The UV index (UVI) is a commonly used indicator of erythral (skin reddening) potential of solar radiation introduced in Canada in 1992 [64]. The UVI is an irradiance scale computed by multiplying the erythral irradiance in Watts/m<sup>2</sup> by 40. The erythral action spectrum for solar radiation has the highest value for below 300 nm and then decreases by a factor of 500 from 300 to 327 nm, and then more slowly out to 39 nm. The UVI multiplies the erythral action spectrum by the solar UV spectrum reaching the earth's



surface, which starts at 290 nm. The UVI peaks from 305 to 310 nm. Satellite instrument data are used to determine two important components of the UVI, total ozone, cloud, and aerosol effects. Surface altitude and latitude are also included. The UVI is given for solar noon.

In Australia, most people have skin pigmentation much lighter than appropriate for the UVB doses. Most people have Anglo-Celtic ethnic backgrounds. In the United Kingdom, the UVI often reaches 6 in summer, and people with Fitzpatrick skin type 2 could be in the sun for 30–60 min without burning (<http://www.weatheronline.co.uk/reports/wxfacts/The-UV-Index.htm>).

As a result, 25(OH)D concentrations for 45-year-old people living in England increase from 37 nmol/L in winter to 75 nmol/L at the end of summer just by going about their lives [28]. However, the Cancer Council of Victoria urges Australians to be “SunSmart” and cover up when the UVI is 3 or higher (<http://www.cancervic.org.au/preventing-cancer/be-sunsmart>). As a result, 25(OH)D concentrations of Australians are not as high as might be expected. A study based on serum 25(OH)D concentrations from women younger than 60 years in the period 1993–2001 found mean peak, monthly peak, and monthly trough values for three locations: South East Queensland (67.0, 75.3, and 54.6 nmol/L, respectively); Geelong region (75.5, 92.5, and 57.1 nmol/L, respectively); and Tasmania (51.1, 62.1, and 40.3 nmol/L, respectively) [106]. The authors’ concluding statement was “Current sun exposure practices and dietary intake do not seem to fully prevent vitamin D insufficiency and deficiency, and consideration should be given to modification of sun exposure advice or pursuing other means to achieve vitamin D adequacy.”

Dermatologists often recommend the shadow rule; i.e., use sun protection when one’s shadow is shorter than one’s height [107]. However, following the shadow rule greatly reduces the production of vitamin D and is associated with increases in risk of melanoma from the higher UVA to UVB ratio [108]. The abstract of the *European Code Against Cancer 4th Edition: Ultraviolet Radiation and Cancer* states: “Excessive exposure from natural sources can be avoided by seeking shade when the sun is strongest, by wearing appropriate clothing, and by appropriately applying sunscreens if direct sunlight is unavoidable. Exposure from artificial sources can be completely avoided by not using sunbeds. Beneficial effects of sun or UVR exposure, such as for vitamin D production, can be fully achieved while still avoiding too much sun exposure and the use of sunbeds” [109]. The Canadian guidelines for the prevention of nonmelanoma skin cancer (NMSC) recommend precautions when the UVI is 3–5, using protection (shade, cover up, wear a hat and sunglasses, use sunscreen) when

the UVI is 6–7, using extra precaution for UVI 8–10, and avoiding the sun more for UVI >11 [110].

Danish sun exposure guidelines recommend seeking shade, wearing a sun hat, wearing protective clothing, or using sunscreen. Adherence to the guidelines regarding seeking shade or wearing protective clothing always or often was associated with approximately 7–10 nmol/L lower 25(OH)D concentrations for adults in spring and autumn [111]. No clear associations were evident for children.

#### 5.4 Sun avoidance because of concern about skin cancer, melanoma

Public interest in the role of solar UV exposure and risk of melanoma and NMSC seems to have been sparked by reports that stratospheric ozone concentrations would be reduced by widespread use of chlorofluorocarbons [112]. In 1980, Australia began its “Slip! Slop! Slap!” campaign to get Australians to avoid the sun for UV indices above 3 [113]. A paper published in the *Journal of the American Academy of Dermatology* in 1982 reviewed the use of sunscreens for protection against the harmful effects of solar radiation [114]. People diagnosed with NMSC are more likely to try to minimize sun exposure through various means, including seeking shade, wearing clothing that exposes less of the body, and using sunscreen [115]. That effect also has been reported in an analysis of cancer rates among participants in the Women’s Health Initiative in the United States [116]. Women diagnosed with NMSC tended to have lower sun exposure in the decade of the study as well as increased risk of several cancers for which UVB reduces risk [117].

#### 5.5 Occupation (outdoors vs. indoors)

People who work outdoors generally have higher 25(OH)D concentrations than those who work indoors. A study in Israel found that outdoor workers had an average daily solar UVB exposure of  $4.4 \pm 1.6$  h, whereas indoor workers had  $0.9 \pm 0.5$  h, resulting in much higher 25(OH)D concentrations for the outdoor workers [118]. Rates of cancer incidence in Nordic countries offer another example of occupation’s effect on 25(OH)D concentrations. Workers in the occupations with most time outdoors, such as farmers, forestry workers, and gardeners, had the lowest rates of cancers for which UVB and vitamin D are associated with reduced risk [119]. Vitamin D production by solar UVB exposure is the only mechanism proposed to explain the link between UVB exposure and reduced cancer risk. A study of professional ballet dancers with mean age of 26 years in the United Kingdom found winter

and summer 25(OH)D concentrations of 37 and 60 nmol/L, respectively [120]. The dancers also had higher injury rates in winter. A study of males in Delhi in August–September found that outdoor, mixed outdoor–indoor, and indoor workers had sun indices of  $12.0 \pm 6.3$ ,  $4.3 \pm 2.2$ , and  $0.7 \pm 0.6$ , respectively, and mean serum 25(OH)D concentrations of  $73 \pm 22$ ,  $48 \pm 14$ , and  $27 \pm 11$  nmol/L, respectively [121]. The sun index was calculated as the product of sunshine exposure in hour/week and fraction of body surface exposed.

Working at night is also associated with reduced 25(OH)D concentrations. A study in Jordan found that female night shift workers had significantly lower 25(OH)D concentrations than day shift workers ( $50 \pm 38$  vs.  $73 \pm 35$  nmol/L) [122]. Male night shift workers had nonsignificantly lower 25(OH)D concentrations than day shift workers ( $55 \pm 25$  vs.  $65 \pm 33$  nmol/L). Also, a review of 10 studies found that shift workers and indoor workers were the occupational groups most likely to have vitamin D deficiency [123].

## 5.6 Outdoor recreational activities

Recreational activities, especially outdoors, can increase 25(OH)D concentrations. Younger people spend more free time outdoors. Analysis of NHANES data from 6370 people older than 18 years from 2003 to 2006 found that 10 min of objectively measured moderate to vigorous activities during the day were associated with an increase in serum 25(OH)D of 0.80 (95% confidence interval (CI) = 0.43–1.20) nmol/L [124]. A study in Italy found that serum 25(OH)D concentrations were about 25% higher for the elderly who regularly engaged in outdoor activities including brisk walking, cycling, gardening, and fishing [125].

## 5.7 Lack of knowledge of the benefits of vitamin D

A study in Hong Kong found that health literacy was directly associated with sunlight exposure, suggesting that health literacy training might be more effective than just providing information about vitamin D and sunlight exposure [126].

An important reason for low 25(OH)D concentrations appears to be lack of knowledge of the benefits of vitamin D, of the risks associated with vitamin D deficiency, and that the sun is an important source of vitamin D. A study of adults aged 20–40 years in Sharjah, United Arab Emirates, found that 39% knew about vitamin D deficiency and 43% of them knew that sunlight is the main source of vitamin D [127]. On the other hand, the elderly are becoming increasingly aware of the role of vitamin D in reducing risk of osteoporotic fractures

and are increasing their oral vitamin D intake. A study in France found that the ratio of 75-year-old women with 25(OH)D concentration <25 nmol/L fell from 69% before 2009 to 35% thereafter [128]. In the United States, analysis of data from NHANES found that age-adjusted mean 25(OH)D concentrations increased from 61 to 63 nmol/L between 1988–94 and 2005–06–67 nmol/L for the period 2007–10 [129]. The increase was attributed to increased vitamin D supplementation.

A study of 208 adult participants in the United Kingdom in 2018 found that 42% answered 4 or 5 of 10 vitamin D questions correctly, while 36% answered 6–10 correctly and 22% answered 1–4 correctly [130]. Forty four percent of the participants reported taking vitamin D supplements. Knowledge score was the strongest predictor of supplement use (odds ratio = 2.5 (95% CI, 1.2–5.3)). The most commonly reported reasons for use were insufficient sun exposure (57%), health benefits (51%), and insufficient amounts from food (46%). Another study conducted online in the United Kingdom during June 17–18, 2019 surveyed public awareness and behavior regarding vitamin D and sunlight exposure in the United Kingdom [131]. Among the findings was that 71% thought that the risks of sun exposure were well promoted versus 22% that thought the benefits of sun exposure were well promoted. Fifty two percent had increased awareness of the risks of sun exposure during the preceding 10 years, but only 24% noticed increased promotion of the benefits of sun exposure during the same period.

Unfortunately, an important reason for lack of knowledge about the health benefits of vitamin D is that the public health and medical systems consider randomized controlled trials (RCTs) to provide the most accurate information on health benefits of pharmaceutical drugs and, by extension, nutrients. Results of observational studies are generally not accepted for setting policies and guidelines. Unfortunately, RCTs of vitamin D for various health outcomes have generally not been designed, conducted, or analyzed properly. As outlined by Heaney [132], the guidelines for nutrients studies (such as for vitamin D supplementation to increase 25(OH)D concentration) include starting with an understanding of the nutrient concentration–health outcome relationship, measure the concentrations for prospective participants, try to enroll participants with low concentrations, supplement with sufficient nutrient to raise concentrations enough to have significant health benefits, and then measure the achieved concentrations. See, also [133]. Unfortunately, most vitamin D RCTs do not restrict their study populations to individuals with vitamin D deficiency [134]. Also, they generally use small vitamin D doses and do not base the analyses on achieved 25(OH)D concentrations. For example, an RCT regarding progression from prediabetes to diabetes



enrolled participants with a mean baseline 25(OH)D concentration of 28 ng/mL and gave participants in the treatment arm 4000 IU/d vitamin D<sub>3</sub> [135]. The hazard ratio for progression to diabetes according to intention to treat was insignificant, 0.88 (95% CI, 0.75–1.04,  $P = .12$ ). However, a subsequent secondary analysis, based on achieved 25(OH)D concentration in the treatment arm, found that the hazard ratio for conversion to diabetes was 0.75 (95% CI, 0.68–0.82) for every 10 ng/mL increase in 25(OH)D concentration above 20 ng/mL to >50 ng/mL [136]. Since it was a secondary analysis, it had little impact on health policy.

In addition, the medical systems such as in the United States consider vitamin D information and supplementation an obstacle to income and profits. As a result, the Disinformation Playbook has been used to discourage the dissemination of information regarding the benefits of vitamin D [137]. The five approaches include promoting bad vitamin D studies, harassing leading vitamin D researchers, manufacturing uncertainty, buying credibility for nonvitamin D approaches to health with academia and professional societies, and capturing government health policy agencies. Examples of these approaches are given in the posted analysis.

### 5.8 Lifestyle factors associated with reduced sun exposure

Both clothing and sun exposure behavior influence the effect of UV on vitamin D concentrations. Extremes of temperature, i.e., both hot and cold, have an impact. Although clothing traditions in equatorial societies are mainly highlighted, the same is true where the temperature is cold. Bedouins and Native Alaskans are traditionally clad from head to toe to prevent themselves from the harsh climate peculiar for their habitats and as such the temperature extremes that they are exposed to. Eventually, irrespective of the hours of sunshine theoretically available for endogenous vitamin D synthesis, the environmental temperature is a major determinant in sun exposure behavior. Studies from sunny countries such as Brazil have found that one of the major contributors of vitamin D insufficiency is seclusion to indoor activity [138].

Furthermore, clothing norms are dictated primarily by cultural following. Conservative societies, as is typical for the Arab world, demand a dress code that restricts the parts of the body exposed. Therefore, even with ample sunshine, type of clothing contributes to vitamin D deficiency. For example, vitamin D deficiency is prevalent among women and neonates in Saudi Arabia because of clothing traditions [139]. Lifestyle choices, such as sun avoidance, indoor work, and covered transport, also may be implicated in the prevalence of vitamin D insufficiency in countries with abundant

sunshine. For women in Morocco, lack of sun exposure and veiled clothing style were the most important factors that influenced hypovitaminosis D [140]. Skin pigmentation, religious belief, and lifestyle were among the major determinants contributing to the prevalence of vitamin D deficiency in South Asia and Southeast Asia [141]. Prevalence of vitamin D deficiency has likewise also been linked to less exposure to sunshine among healthy schoolchildren in central Ethiopia [142] and among Iranian adolescents [143]. A study listed the aforementioned as the major contributors in Cambodian women, despite their living close to the equator [144].

Pale skin has historically been prized as beautiful in China, and that concept is widespread in other Asian countries, such as India (<http://asiasociety.org/blog/asia/china-long-tradition-dodging-sun-photos>). Thus, aesthetically the preference of fair complexion has also retarded sun exposure in several Asian communities [145].

Cultural beliefs tend to prevail independently of country of domicile—particularly for the Asian diaspora. A study on South Asian women residing in Auckland, New Zealand, reported that deliberate sun avoidance and an indoor lifestyle were the major causes of hypovitaminosis D [146]. More sun protection behavior, shorter sun exposure on weekends, and less acculturation to the Australian lifestyle all were associated with vitamin D deficiency in East Asian women living in Sydney, Australia [147]. Primary healthcare patients of African and Asian origin in Sweden were at high risk of vitamin D deficiency [148]. A study of East Asian women living in Australia reported the following: “These women reported a number of cultural factors related to their attitudes and behaviors regarding sun exposure. They expressed preference for fair skin, a tradition of covering skin when outdoors, and no sunbathing culture. They believed that fair skin was more beautiful than tanned skin. They reported that beauty was the reason for active avoidance of sunlight exposure. Although they reported knowledge of the need for sun avoidance due to skin cancer risk, few reported knowledge about the benefits of sun exposure for adequate vitamin D levels” [147,149].

### 5.9 Migration/migrants

The global migrant/refugee crisis will probably exacerbate vitamin D deficiency. An increasing number of countries were recently confronted with hundreds of thousands of immigrants. In Germany, for example, the number of immigrants of European origin increased 1.3-fold between 2008 and 2015 [104,105]. At the same time, the number of immigrants from Africa increased 1.6-fold, whereas that from Middle East and Asia

increased 1.9-fold. Furthermore, the second generation from overseas-born migrants should be also considered a group at high risk for vitamin D deficiency because of darker skin pigmentation than the host population and reduced rate of full assimilation to the host society and its habits (lifestyle and diet). Dark-skinned immigrants in Europe have a significantly increased rate of rickets [150]. In the United States, African Americans have significantly lower 25(OH)D concentrations than European Americans and, therefore, increased risk for many types of disease [151].

Compared with the migrants' sunny homelands, most of the host countries (high income) are at higher latitudes with reduced efficacy of UVB, cloudy skies, pollution, low average temperatures, and short summer season (except Australia). As a consequence, clothing effectively prevents skin synthesis of vitamin D because of weather conditions or cultural and religious reasons. Unfortunately, the dietary preferences of migrants who relocated to higher latitudes may intensify their risk for vitamin D deficiency. Only a few natural food products are rich in vitamin D, so vitamin D-fortified foods and vitamin D supplements can serve as an alternative source. However, because of dietary preferences or economic status, immigrants may not consume commonly fortified staple foods or supplements [152]. Therefore, displacement from tropical regions to high-latitude countries puts immigrants at even greater risk of vitamin D deficiency than in their country of origin.

As shown by Mughal and colleagues [153], prolonged breastfeeding without maternal vitamin D supplementation to benefit the infant is another problem within immigrant societies that increase the risk of vitamin D deficiency and its consequences. Thus, exclusively breastfed infants consuming 750–1000 mL of breast milk per day from vitamin D-deficient mothers fail to receive the 10 µg/day of vitamin D needed to at least prevent bone mineralization defects [153]. A clinical trial showed that nursing women supplemented with 6400 IU/day of vitamin D<sub>3</sub> delivered 400 IU/day vitamin D<sub>3</sub> to the nursing infants [154].

In a study from Italy, severe vitamin D deficiency [25(OH)D < 25 nmol/L] was noted in 76% of migrant newborns and 48% of migrant mothers [155]. Both migrant newborns and migrant mothers had very low 25(OH)D concentrations ( $18 \pm 14$  and  $30 \pm 17$  nmol/L, respectively). Among the studied mother–infant pairs, a linear decrease of 25(OH)D concentrations was observed with increasing skin pigmentation (phototype I,  $42 \pm 18$  nmol/L, vs. phototype VI,  $18 \pm 10$  nmol/L;  $P < .0001$ ) [155]. For data analyzed by country of origin, host country newborns from Italy had 25(OH)D

concentrations higher than all migrant groups ( $P < .0001$ ) such as North African, African, Asian, Central–South American, and East European. The same results were found in host country mothers, and North African mothers and their offspring had the lowest 25(OH)D concentrations of  $22 \pm 11$  and  $13 \pm 10$  nmol/L, respectively [155]. In the Netherlands, 25(OH)D concentrations <50 nmol/L were identified in 82% of Surinamese, 92% of Turkish, and 93% of Moroccan pregnant women compared with 28% of native Dutch women [156].

In Belgium, 90% of Moroccans and 77% of Congolese had serum 25(OH)D concentrations <50 nmol/L [157]. In Norway, 92% of Pakistanis had 25(OH)D concentrations below 50 nmol/L [158], and 81% of newly arrived immigrants from the Middle East, 75% from South Asia, and 73% from Africa had 25(OH)D concentrations lower than 50 nmol/L [159]. Furthermore, from aforementioned regions, approximately one-third had 25(OH)D < 25 nmol/L [160]. Vitamin D deficiency appeared common among Pakistani immigrant children in Denmark [161]. Somali immigrant women had a high prevalence of vitamin D deficiency, defined as 25(OH)D < 50 nmol/L, with rates of 90% in Norway [162] and Finland [163].

A recent metaanalysis of dark-skinned migrant populations showed that immigrants from the extended Middle East and sub-Saharan Africa had a high prevalence of vitamin D deficiency (65% and 56%, respectively) [164]. Refugees are considered particularly at risk for vitamin D deficiency because of staying indoors to avoid potential harm from conflict in native countries and dangers associated with refugee camps [165]. Longer time spent in the host country is an additional risk factor for vitamin D deficiency, as suggested in the literature. In one study, the length of time living in Melbourne, Australia, was associated with increased risk of vitamin D deficiency (defined as 25(OH)D < 25 nmol/L) in African migrants, with a prevalence of 77% for those living >2 years in Melbourne compared with a prevalence of 38% for those living less than 2 years in that city [166]. The aforementioned phenomenon might be linked to asylum seekers dressing more conservatively since immigrating to Australia as well as reporting apartment-style accommodation and reduced time spent outdoors, further reducing sun exposure from that before they immigrated [166].

In migrant and refugee subpopulations, major risk factors for vitamin D deficiency include darker skin, Muslim religion, full body–covering clothing, longer stay in host country, decreased daylight exposure, living in an urban environment, and coming from a socioeconomically disadvantaged background. Prevention

programs with vitamin D supplementation should be considered in host countries, and migrants/refugees at high risk should be educated, screened, and monitored for vitamin D deficiency.

## 6. Predicting vitamin D deficiency

Diffey developed a model to estimate 25(OH)D concentrations from UV exposure in summer in the United Kingdom. He found that then-current advice to spend 10–20 min in the sun did little to boost overall 25(OH)D concentrations [167]. He extended that model later to include oral intake, finding that a combination of increased oral vitamin D intake in winter and increased summer sun exposure could improve vitamin D status for the adult British population [168]. His models were used to develop an integrated predictive model of population 25(OH)D concentration for Ireland on the basis of UVB data and vitamin D from food sources (4.4 µg/day or 180 IU/day). That model predicted well the 18%–19% of the population with 25(OH)D concentration <30 nmol/L in winter [169]. Evidently, 25(OH)D has a very slow decay rate, as shown by the fact that elderly patients who received vitamin D<sub>3</sub> in the form of fortified bread achieved a 25(OH)D concentration of 127 nmol/L, which decayed to 65 nmol/L after 1 year without the bread and 48 nmol/L at the end of 3 years [170].

Some researchers tried to predict vitamin D deficiency on the basis of several demographic characteristics and lifestyle factors. A cross-sectional study of 644 60- to 84-year-old participants in Australia found that use of “time outdoors, physical activity, vitamin D intake and ambient UVR, and inversely correlated with age, BMI, and poor self-reported health status” explained 21% of the variance in 25(OH)D concentration [171].

Another study in Amsterdam involved 1509 elderly Dutch participants in the development sample and 1100 in the validation sample [172]. “The final model for the prediction of vitamin D concentrations <30 nmol/L consisted of 10 predictors: older age, smoking, alcohol consumption (<13 drinks/wk), season, no vitamin supplement use, no bicycling, no gardening, medication use, limitations in the use of own or public transportation, and the inability to remember the present year, etc. The final prediction model for serum 25(OH)D < 50 nmol/L consisted of the following 13 variables: older age, sex (female), BMI (>30), smoking, alcohol consumption (<13 drinks/wk), season, no vitamin supplement use, no bicycling, no sporting, no gardening,

medication use, poor appetite, and without a partner.” The resulting model was sensitive to the upper 25(OH)D concentration cutoff. Table 4 in that article showed that the lower the cutoff concentration, the lower the specificity and the higher the sensitivity. Analysis of data from the Nurses’ Health Study and Nurses’ Health Study II in the United States found that for women, >10 g/day alcohol consumption raised 25(OH)D concentration by about the same amount as 7.5 µg/d (300 IU/d) vitamin D<sub>3</sub> intake [173].

A recent study based on analysis of 201 healthy individuals between the ages of 20 and 40 years identified male sex ( $P < .001$ ), taking 50,000 IU vitamin D<sub>3</sub> supplement monthly ( $P < .001$ ), and lower waist circumference ( $P = .02$ ) were identified as effective factors in increasing serum 25(OH)D levels [174]. It is difficult to predict serum 25(OH)D concentration with respect to vitamin D supplementation due to several factors including solar UVB exposure, BMI, dietary sources of vitamin D, and genetics. A figure posted at [Grassrootshealth.net](https://www.grassrootshealth.net/document/serum-25ohd-vs-vitamin-d-supplement-intake/) based on values for their volunteer cohort shows that while 25(OH)D concentration rises in a nonlinear manner from 100 nmol/L with no vitamin D supplementation to 240 nmol/L for 20,000 IU/d, there is considerable variability for any supplementation amount (<https://www.grassrootshealth.net/document/serum-25ohd-vs-vitamin-d-supplement-intake/> access ed March 22, 2022).

The question is often raised whether it is worthwhile to measure 25(OH)D concentrations especially given cost considerations. One analysis suggested that it is when targeted at people likely to have vitamin D deficiency such as those with chronic diseases, having dark skin living at higher latitudes, and the elderly [175]. Regarding darker skin, see Ames et al. [151].

## 7. Nonvitamin D benefits from solar UV exposure

There is now good evidence that there are important health benefits from solar UV exposure in addition to vitamin D production. The primary mechanism is liberation of nitric oxide (NO) from intracutaneous nitrogen compounds, primarily nitrate, by UVA [176,177]. NO is known to reduce the risk of cardiovascular disease [178], and fight viral infections [179], among other things. In fact, it appears to explain the seasonality of many health outcomes with highest rates in winter and lowest in summer [180]. Solar UVA has been found to reduce risk of autoimmune diseases [181,182], blood pressure

[183], metabolic dysfunction [184], and COVID-19 [185]. The COVID-19 study was done in winter in Italy, the United Kingdom, and the United States, in locations where solar UVB doses were so low that vitamin D could not be produced. Thus, the additional health benefit of NO liberation should be considered an added incentive to spend time in the sun. For those who have limited access to sun exposure in any season, a UV lamp can be used to produce both vitamin D and NO [186].

## 8. Conclusion

Although solar UVB exposure is the most important source of vitamin D for most people, many factors affect 25(OH)D concentrations related to solar UVB doses. Some of these factors are physical, relating to solar, atmospheric, and surface properties and time of day. Others are related to lifestyle, such as time spent outdoors, clothing worn, and use of sunscreen. These factors are subject to societal factors, including attitudes toward sun exposure. Genetic factors play a role in skin pigmentation and are involved in the metabolism and transport of vitamin D. Since solar UVB exposure is subject to physical, cultural, and personal restraints, vitamin D food fortification and vitamin D supplementation would be required to have everyone avoid vitamin D deficiency [8,12,169].

## 9. Summary points

- Solar UVB exposure is the most source of vitamin D.
- Many factors affect 25(OH)D concentrations associated with solar UVB exposure including solar elevation angle, duration of sun exposure, amount of skin exposed, genetics, age, body mass, skin pigmentation including that from tanning.
- In Europe, the mean 25(OH)D concentration is near 68 nmol/L in summer for all countries; in winter there is a U-shaped relationship between 25(OH)D concentration and latitude, with lowest concentrations near 45–50 degrees N latitude. For all latitudes, 25(OH)D stored in muscles is recirculated in winter; concentrations are higher at the lower latitudes in winter due to longer duration of vitamin D production, and higher at the higher latitudes due to greater consumption of dietary sources of vitamin D including ocean fish and meat, as well as taking vitamin D supplements.
- An important reason for low 25(OH)D concentrations is that the medical and health systems have not embraced vitamin D supplementation for nonskeletal health effects due to the paucity of successful vitamin D supplementation RCTs. The failure of most vitamin

D RCTs is due to poor design, conduct, and analysis tied to not basing the trials on 25(OH)D concentration instead of vitamin D dose.

- There are additional health benefits from solar UV exposure due to nonvitamin D effects of UVB and increases in NO concentrations in the serum from UVA exposure.

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# Vitamin D supplement use as a public health strategy to augment diet and sustain population adequacy

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## OBJECTIVES

- Describe inadequate vitamin D status as a worldwide problem present in countries where sun exposure and/or dietary sources are not widely used.
- Demonstrate that dietary supplements are used by otherwise healthy individuals to fill dietary gaps and vitamin D supplement users show better status than nonusers.
- Discuss, using Canada as a case study, the situation where vitamin D supplements are recommended by the Ministry of Health yet uptake in their use rose only modestly over a decade (2004–2015).
- Describe and discuss reasons for low uptake of vitamin D supplementation use.
- Present rationale for concluding that vitamin D supplement use cannot be an effective population health strategy unless better government guidance is provided and regulatory policies governing staple food fortification are established.

## 1. Introduction

Vitamin D deficiency is a recognized problem worldwide, and there is a pressing need to address ways in which adequate vitamin D status can be maintained in populations [1–3]. While sun exposure (providing ultraviolet B [UVB] rays) should be able to satisfy our need for vitamin D through dermal synthesis, there are environmental factors and personal characteristics that prevent or impede synthesis in the skin, and these are discussed in [Chapter 56](#). Many countries, especially those having prolonged “winter” during which time UVB levels are low or in countries where there is a “latitude–skin color mismatch” of people living there [4], must rely on dietary vitamin D to sustain adequate circulating concentrations of 25(OH)D. However, naturally rich food sources of vitamin D, which are primarily marine mammals and fish, are infrequently available or not consumed by choice. A major strategy to correct population micronutrient inadequacy, including vitamin D, involves the fortification of food to increase intake, a strategy that is covered in detail in [Chapter 58](#). As described in the following, national survey data show that total vitamin D intakes from natural food sources and even from foods that have been fortified do not meet current dietary recommendations for

vitamin D. Thus, another strategy is to encourage supplementation with vitamin D to fill the dietary gap. This chapter describes how current efforts to maintain adequate vitamin D intakes in most of the population can be enhanced by supplement use. A public health policy for safe, targeted, and effective use of vitamin D supplements needs to cover the majority of the population in an equitable way, which can present many challenges. The focus of this chapter questions: “Is it possible to maintain vitamin D adequacy in the population using supplementation?”

Table 57.1 shows the use of vitamin D supplements in three specific areas of health and medical care, which is an accepted and widely prescribed practice [6]. Many countries have recommendations of vitamin D supplement use for breast-fed infants (see Chapter 33), during pregnancy (see Chapter 32), and for disorders of liver (see Chapter 79), kidney (see Chapter 81), and gastrointestinal tract (see Chapter 97), which directly affect vitamin D metabolism as well as numerous diseases that are dependent upon vitamin D status. The use of vitamin D supplements in these diseases and medical

**TABLE 57.1** Categories of vitamin D supplement use to distinguish various supplement recommendations in the literature.—cont’d

Type of supplement use <sup>a</sup>	Criteria <sup>a,2</sup>	Comments
Recommendations for treatment of vitamin D deficiency	<ul style="list-style-type: none"> <li>Come from professional scientific societies.</li> <li>Are disease-specific vitamin D recommendations, developed mainly as an addendum to therapy of the disease.</li> <li>May be a prevention strategy for the disease and its clinical complications.</li> </ul>	osteoporosis, menopause, kidney disease, geriatrics, dentistry etc.
	<ul style="list-style-type: none"> <li>Patients with a laboratory confirmed vitamin D deficiency.</li> <li>Age- and body weight-dependent therapeutic dosage should be administered.</li> <li>Protocols are regional or national treatment recommendations.</li> <li>Treatment duration is typically 1–3 months and involves bolus dosing.</li> </ul>	Patients with a severe liver dysfunction or chronic renal disease are the only groups that require the use of activated vitamin D metabolites. Patients with intestinal malabsorption may require large oral doses or intramuscular injections.

<sup>a</sup>Source: Pludowski et al. 2018 [6]; Gibson, 2022 [8].

care examples is specific to individual patient’s need. In contrast, this chapter specifically addresses the use of vitamin D supplements by the general population over the age of 1 year for whom vitamin D supplementation can help to maintain status (for example, during winter months) or raise vitamin D status over a threshold for optimal health in an otherwise healthy individual. Unless specified, it is assumed supplements are in the form of the more widely available vitamin D<sub>3</sub> and are taken daily.

Recent evidence of the role of vitamin D supplementation in reducing risk of chronic disease and acute infections has sparked interest and research toward establishing higher thresholds of circulating 25(OH)D that are associated with the reduction in disease risk. The role of vitamin D supplement use in decreasing prevalence of inadequacy of vitamin D in the general population can be assessed using national intake and status data that allow monitoring of supplement use in

**TABLE 57.1** Categories of vitamin D supplement use to distinguish various supplement recommendations in the literature.

Type of supplement use <sup>a</sup>	Criteria <sup>a,2</sup>	Comments
Recommendations for general population	<ul style="list-style-type: none"> <li>Intake recommendations for populations that are released by authoritative bodies with subsequent government approval.</li> <li>Levels of intake needed to maintain health in already healthy individuals.</li> <li>Based on the typical dietary pattern of the country.</li> <li>Include nutrient reference values for minimum (e.g., RDA and maximum (i.e., upper) levels of intake.</li> </ul>	Institute of Medicine’s dietary reference intakes (2011) shown in Table 57.2. For a comparative list, see bouillon [5].
Recommendations for patients suffering from a disease	<ul style="list-style-type: none"> <li>Vitamin D supplementation guidelines that are disease-specific.</li> </ul>	Many disease-specific national or international guidelines related to

sustaining adequacy. Government guidelines providing citizens with basic education to encourage safe, targeted, and effective population use of vitamin D supplements are critical to the success of such a public health policy. The barriers we discuss center around inconsistent and ineffective messaging by governments and a lack of consensus from healthcare providers on the need for supplement use. Many of the examples we draw from are data from Canada, a country with a lengthy vitamin D winter 5–10 months long for most Canadians who live between latitude 42° and 84°N. It is a country that has mandatory vitamin D fortification of two staple foods, fluid cow milk, and margarine. As well, Canadians have access to regulated supplements that are safe, inexpensive, and available in pharmacies and health food stores. We show that despite these opportunities, the vitamin D status of many Canadians needs improvement.

## 2. Thresholds for 25(OH)D

To relate vitamin D intake to status, i.e., serum 25(OH)D measures, it is common to have a two-tier set of terms. “Deficiency” (sometimes called insufficiency) is defined as a level of serum 25(OH)D not able to provide protection against rickets/osteomalacia and osteoporosis, which represent the traditional outcomes of vitamin D deficiency. Most countries with published dietary vitamin D intake guidelines have set this level as < 50 nmol/L (20 ng/mL) [5], and there is near global consensus. To be above this cutoff is to achieve “sufficiency,” which is the term we use in this chapter for the lowest threshold of vitamin D status that is related to a health outcome. Each country-based report provides an intake called the recommended dietary allowance (RDA) or an equivalent dietary intake goal, which is the amount to maintain this level of sufficiency (50 nmol/L threshold) for most of the population in the absence of sun exposure (Table 57.2). The intake recommendation is intended as a guide for health policy decisions in that country for such initiatives as food fortification, food labels, and institutional feeding in schools, hospitals, and nursing homes. The RDA is the amount that an already healthy person needs to maintain health (e.g., in Canada and the United States, the RDA is to maintain bone health) and is, in most cases, calculated as the amount that will allow most of the population to achieve the adequacy threshold [7]. The EAR or estimated average requirement is the daily intake required to achieve circulating 25(OH)D concentrations of 40 nmol/L, which is the amount of vitamin D intake intended to meet the needs of half the population. For the purposes of this chapter, we refer to the RDA as

**TABLE 57.2** Institute of medicine’s (2011) nutrient reference values [7]. For the population aged 1 year and over, vitamin D set by Canada and the United States that have been adopted by other countries.

Age/Sex group	EAR <sup>a</sup> μg (IU)/day	RDA <sup>b</sup> μg (IU)/day
1–3 y M&F	10 (400)	15 (600)
4–8 y M&F	10 (400)	15 (600)
9–18 y M&F	10 (400)	15 (600)
19–70 y M&F	10 (400)	15 (600)
Over 70 y M&F	10 (400)	20 (800)

<sup>a</sup>EAR, estimated average requirement, is based on achieving 40 nmol/L serum 25(OH)D; it is intended to meet the requirement of half the population.

<sup>b</sup>RDA, recommended dietary allowance, is based on achieving 50 nmol/L serum 25(OH)D; it meets the needs of most of the population.

the intake guideline needed to sustain adequate vitamin D status, as this amount is the goal for individuals.

An “optimal” concentration of 25(OH)D is the second term often used when assessing vitamin D status. Here, the threshold represents the measure of circulating 25(OH)D based on emerging evidence of this threshold being associated with the reduction in risk of disease. Table 57.3 illustrates various health conditions established in different countries and the associated serum 25(OH)D threshold for optimal health. Among various countries, the lowest threshold is the concentration associated with the prevention of severe deficiency (rickets and osteomalacia) and is the value set by the United Kingdom’s Scientific Advisory Committee on Nutrition (SACN) [9]; the United Kingdom notably has a high prevalence of rickets [10]. Next, at 50 nmol/L, is the threshold for bone health that was initially derived by the IOM in 2011 [7]. Some optimal thresholds shown in Table 57.3 are derived from an analysis of research studies showing significant reduction of risk for specific preventable illnesses and death, and most are attributed to vitamin D actions that include autocrine, paracrine, and intracrine functions—extraskelatal functions (see Chapter 9). Of these optimal thresholds, most are arrived at by combining results from multiple research designs appropriate to establishing requirements for a nutrient’s health effects [11], while one threshold, that of a reduction in autoimmune diseases, is based on results from a recent large randomized controlled trial called VITAL [12].

For the purposes of this chapter, discussion of vitamin D supplement use relies on results from research that compares outcomes of supplement users compared with nonusers in terms of achieving adequacy of vitamin D status compared with the requirement intake level and/or the deficiency threshold 25(OH)D concentration of the country. Most countries use the adequate

**TABLE 57.3** Serum 25(OH)D concentration thresholds for various health outcomes.

Outcome	25(OH)D nmol/L
Severe deficiency <sup>a</sup>	25
Bone health <sup>b</sup>	50
Alzheimer's disease and dementia <sup>c</sup>	62.5
Osteomalacia <sup>d</sup>	75
All-cause mortality <sup>c</sup>	75
Myocardial infarction <sup>c</sup>	75
Colorectal cancer <sup>c</sup>	75
Cardiovascular disease <sup>c</sup>	75
Hypertension <sup>c</sup>	100
Preterm delivery <sup>c</sup>	100
Autoimmune disease <sup>e</sup>	100
SARS-CoV-2 infection <sup>c</sup>	125
Diabetes mellitus type 2 <sup>c</sup>	125
COVID-19 mortality <sup>c</sup>	150
Breast cancer <sup>c</sup>	150

<sup>a</sup>SACN [9].<sup>b</sup>IOM 2011 [7].<sup>c</sup>Grant et al., 2022 [11].<sup>d</sup>Priemal et al., 2010 [13].<sup>e</sup>Hahn et al., 2022 [12].

Values taken from

vitamin D status threshold for bone health, which is 50 nmol/L [5]. Some researchers, however, may compare outcomes at the higher thresholds depicted in Table 57.3, based on recent advances in vitamin D research. For example, Ames et al. [4] consider use of 4000 IU (100 µg) to achieve a 25(OH)D concentration of at least 75 nmol/L for African Americans, and the VITAL trial used 2000 IU (50 µg) to achieve a 25(OH)D level of (on average) 100 nmol/L for reduction in risk of several chronic diseases [12,14].

### 3. Country-level guidelines for supplements use

Supplements, when used for prevention, fill the gap between intake and requirement that may exist in a country that cannot be filled by other means such as fortification or sun exposure guidelines. Nations provide dietary guidelines so that the population can choose healthy yet culturally appropriate foods to meet nutritional needs. However, for vitamin D, food choices in countries that have no fortification or limited foods that are fortified, the selection pool of foods to provide natural vitamin D is small.

Some countries have provided guidance through their Ministries of Health or equivalent government department on the use of vitamin D supplements by the general population (Table 57.4). Finding these recommendations limited us to searchable statements that may not have identified all countries with vitamin D supplement guidance; although possibly incomplete, the list provides examples of varying government advice. Two countries (the United Kingdom, Hong Kong-SAR) specifically target lack of sun exposure for the general population as the main barrier to sustaining vitamin adequacy. For the United Kingdom, it recognizes that winter is a time when vitamin D synthesis is not possible, while Hong Kong, situated close to the equator, recognizes that being indoors or wearing clothes that cover most of the skin can lead to vitamin D deficiency. Four countries make supplementation recommendations specific to age: Canada (age 2–50 years and >50 years), Ireland and Switzerland (>65 years), and Finland (<18 years and “older people”). All but Hong Kong recommends a specific amount for supplementation: Canada recommends 400 IU (10 µg) daily, which was set in 2007; the more recent Swiss recommendation (released 2022) is 800 IU (20 µg); the Finnish recommendation for supplement use alone is 20 µg; for Ireland, a daily amount of 15 µg (600 IU) is specified; and for the United Kingdom, the supplement amount is 10 µg (400 IU) for all ages.

For recommendations shown in Table 57.4, it is not known if governments have monitored adherence to this guidance or plan to implement monitoring of the impact of these supplement recommendations in the future. In Canada, the initial recommendation (shown in the footnote) was implemented in 2007 as a feature of a new version of Canada's Food Guide, which was replaced in 2019. In the 2007 Canadian Food Guide, the vitamin D supplement recommendation was highly visible and is believed to have influenced supplement use. Canadian national surveys show an increase in supplement use by adults over 50 years of age between 2004 and 2015 [15]. For Canadian adults over 50 years of age, the percent who use D-supplements rose from about 45% to 55% for women over 50 years and from 30% to 35% for men over 50 years. These gains are quite modest considering the proportion of older adults taking a supplement falls short of the Canadian recommendation that everyone in this age group (>50 years) needs a supplement. The new recommendation for Canada (Table 57.4) better reflects current RDAs as indicated for everyone, not just those over 50 years may not get sufficient dietary vitamin D from foods alone.

The recommended supplement amounts made by the various governments (Table 57.4) are within the range of the RDA or equivalent values set to prevent severe deficiency or to ensure bone health with a circulating

**TABLE 57.4** Examples of advice and/or recommendations for citizens of a country to take a vitamin D supplement issued by the ministry of health or equivalent government body. Excludes Recommendations during pregnancy or for breast-fed infants.

Country	Organization	Recommendation	Reference or source
Canada	Health Canada <sup>a</sup>	"Include vitamin D from foods or a supplement every day. If you are between 2 and 50 years old: eat foods that contain vitamin D every day or take a daily supplement containing 400 IU (10 µg) of vitamin D. If you are 51 years of age and older: Take a daily supplement containing 400 IU (10 µg) of vitamin D; you can continue to eat foods that contain vitamin D as part of healthy eating."	<a href="https://www.canada.ca/en/health-canada/services/nutrients/vitamin-d.html#a2">https://www.canada.ca/en/health-canada/services/nutrients/vitamin-d.html#a2</a>
United Kingdom	National Health Service	"All children aged 1–4 years old should be given a daily supplement containing 10 µg of vitamin D" "Because it's difficult for people to get enough vitamin D from food alone, everyone (including pregnant and breastfeeding women) should consider taking a daily supplement containing 10 µg of vitamin D during the autumn and winter."	<a href="https://www.nhs.uk/conditions/vitamins-and-minerals/vitamin-d/">https://www.nhs.uk/conditions/vitamins-and-minerals/vitamin-d/</a>
Finland	Finnish Food Authority	"A daily vitamin D supplement is recommended all year round for all those living in Finland who are pregnant, breastfeeding or aged under 18 years, and those who do not use vitamin D fortified products or fish regularly ... people who spend average amounts of time outdoors in the summer months need to get about 10 µg/day of vitamin D from their diet or as a dietary supplement through the year .... Older people, and also younger people who spend little time outdoors, should get up to 20 µg/day of vitamin D ...."	<a href="https://www.ruokavirasto.fi/en/themes/healthy-diet/nutrients/vitamin-d/">https://www.ruokavirasto.fi/en/themes/healthy-diet/nutrients/vitamin-d/</a>
Ireland	Department of Health	"The Department of Health is today advising that adults aged 65 and older take a vitamin D supplement to ensure they get the essential vitamin D needed for bone and muscle health." "The recommendation is that adults aged 65 and older take a vitamin D supplement of 15 µg (15 µg) every day to ensure they get the essential vitamin D needed for bone and muscle health."	<a href="https://www.gov.ie/en/press-release/7d595-new-advice-on-vitamin-d-supplement-for-people-aged-65-years-and-older/">https://www.gov.ie/en/press-release/7d595-new-advice-on-vitamin-d-supplement-for-people-aged-65-years-and-older/</a>
New Zealand	Ministry of Health	"Some people with a high risk of vitamin D deficiency may need to take a vitamin D tablet. Talk to your GP, dietitian or lead maternity carer if you're concerned."	<a href="https://www.health.govt.nz/your-health/healthy-living/food-activity-and-sleep/healthy-eating/vitamin-d">https://www.health.govt.nz/your-health/healthy-living/food-activity-and-sleep/healthy-eating/vitamin-d</a>
Hong Kong special administrative region	Family Health Service of the Department of Health.	"People at risk of not getting enough vitamin D ... [including] ... People who spend most of their times indoors; People who keep all skin covered up by clothing .... If you are concerned, please consult your doctor to assess the need for vitamin D supplements."	<a href="https://www.fhs.gov.hk/english/health_info/child/30078.html">https://www.fhs.gov.hk/english/health_info/child/30078.html</a>
Switzerland	Swiss Federal Food Safety and Veterinary Office (BLV)	"From the age of 65, it is recommended to take a dose of 800 IU of vitamin D per day, as form of drops or capsules. Talk about it beforehand with your doctor."	<a href="https://www.dsm.com/human-nutrition/en/talking-nutrition/switzerland-takes-positive-steps-towards-supporting-vitamin-d-status-in-older-adults.html">https://www.dsm.com/human-nutrition/en/talking-nutrition/switzerland-takes-positive-steps-towards-supporting-vitamin-d-status-in-older-adults.html</a>

<sup>a</sup>These recommendations were released May 2022. Prior to this date, the recommendations were as follows: "If you are over 50 years old, Health Canada recommends that you take a daily vitamin D supplement of 400 IU (equivalent to 10 µg)" and "Speak to your healthcare provider about taking a vitamin D supplement if you think you are not getting enough of it."



25(OH)D threshold goal of 50 nmol/L [5]. However, these intake recommendations would not be enough to reach any of the optimal levels of 25(OH)D for reducing risk of many chronic conditions shown in Table 57.3. As it is not common to have serum 25(OH)D analyzed in the general population, then dietary intakes of vitamin D are needed for guidance to estimate the likelihood of maintaining adequate D status. These RDA and equivalent values given as recommended levels of intake for vitamin D supplements should be as up to date as possible and must be relevant to current conditions of the targeted population. A case in point illustrating the need for effective intake advice is shown by the vitamin D supplement level recommendations issued by the Minister of Health in the United Kingdom from September 2020 to February 2021 in response to the early phase of the COVID-19 pandemic. The United Kingdom through the action of the Minister of Health made 400 IU supplements available at no cost to vulnerable populations during the pandemic lockdowns [16]. Nonetheless, many scientists believed that this low level of vitamin D was insufficient to meet increased immune protection that could be afforded by vitamin D and requested a higher dosage be made available for free to these vulnerable groups [17], but no increases have occurred and the debate continues.

#### 4. Supplement use improves vitamin D status: Canada as a case study

Canada is a country with mandatory vitamin D fortification of two staple foods, cow's milk and margarine, as well as voluntary fortification of other foods. Yet Canada has a high prevalence of vitamin D status inadequacy from those relying on foods alone (i.e., supplement nonusers). In 2015, mean vitamin D intake in the population not using supplemental vitamin D was only 4.2 µg/d (168 IU), and there was an overall 96.5% prevalence of poor vitamin D status measured in a national survey representative of the population for those 1 year and over as reported by the Canadian Community Health Survey (Table 57.5) [15]. Prevalence of inadequate vitamin D status or intake is a calculation of the proportion of the population that does not meet the RDA for intake or threshold for adequate status, 15–20 µg/d (600–800 IU) and 50 nmol/L 25(OH)D, respectively. The 2015 Canadian Community Health Survey (CCHS) measured dietary intakes year-round; thus, it collected over the winter months during which time the population in Canada experiences “vitamin D winter” for at least 5 months. As shown in Table 57.5, there were no differences in prevalence of intake inadequacy by age or sex for the nonusers, which is unusual considering differences in the amount of total food

intake between adults and children. An explanation can be gleaned from data that were analyzed in a previous CCHS conducted in 2004 when vitamin D intakes were no different from the 2015 daily intakes. In examining food sources of vitamin D by age groups, it was found that the children consumed more milk as a source of vitamin D, while adults ate more meat (including fish) and alternatives (including eggs) than children [15]. Hence, the vitamin D food sources of Canadians differed by age, with children reliant on a vitamin D–fortified food source and adults consuming more natural and unfortified foods containing vitamin D. Both age groups, however, fell short of reaching recommended intakes.

When the Canadian supplement users (33% of the population) were examined for vitamin D intakes in the 2015 CCHS, mean vitamin D intake was 14.1 µg/d (564 IU) and prevalence of inadequate intake at 14.1%, was markedly lower than for nonusers (Table 57.5) [15]. In contrast to the intakes from food alone by nonusers, intakes from foods alone and intakes from foods plus supplements varied by age and gender. For foods alone, intakes by supplement users were highest in males aged 14–30 years suggesting that this group was more health conscious than those who did not use supplements. For intakes from food and supplements together, the lowest vitamin D intake was 16.7 µg/d (668 IU) in children aged 1–8 years and the highest was 41.2 µg/d (1648 IU) in women ≥71 years. These survey data provide evidence that supplement use combined with mandatory food fortification enables more Canadians across age to meet the recommended intake levels of vitamin D.

The 2015 Canadian national nutrition survey showed only a modest increase of 5% in prevalence of supplement use from the 2004 intake survey. In 2015, there were 33% of the population 1 year and over taking a vitamin D supplement compared with 28% in 2004 [15]. The variability in proportion of supplement users in the population by age and gender is shown in Fig. 57.1. Those in the two oldest age groups had the highest percentage of use compared with younger subjects of the same sex in both surveys, and the increase in use by these older age groups was the main reason for the overall 5% rise in prevalence of supplement use of Canadians over 1 year of age. The 2007 Canada Food Guide recommended vitamin D supplement use that specifically targeted those over 50 years of age, and this recommendation has remained on the Health Canada website until May 2022 (Table 57.4). The recommended dose remains at 10 µg/d (400 IU); this supplement amount filled the gap between intake from food (200 IU) in 2004 and the vitamin D recommendations of 2007, which were 400 IU for those over 50 years and 600 IU for those over 70 years [18]. Higher RDAs for all age groups over 1 year of age, of 15 µg/d (600 IU)

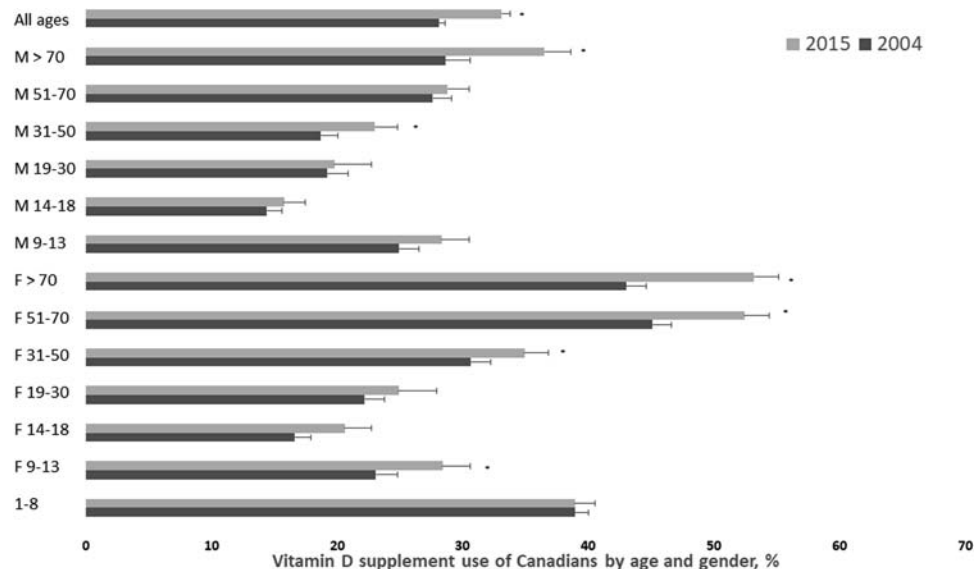
**TABLE 57.5** Comparison of prevalence of inadequacy between supplement users and nonusers using nationally representative usual intake data from the Canadian Community Health Survey 2015.

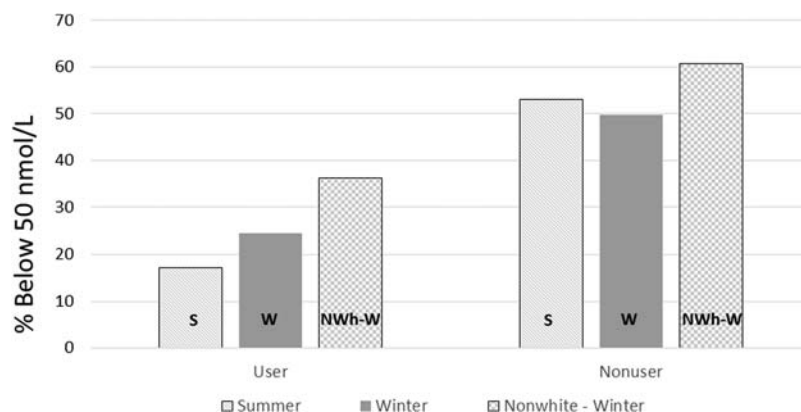
Age and sex groups	Supplement nonusers		Supplement users		
	Vitamin D from all foods	Prevalence of inadequacy <sup>a</sup>	Vitamin D from all foods	Vitamin D from all foods + supplement	Prevalence of inadequacy <sup>a</sup>
F&M 1–8 y	µg/day 4.1 ± 0.2	% 99.0 ± 0.4	µg/day 5.3 ± 0.3	µg/day 16.7 ± 1.1	% 38.0 ± 3.4
F 9–13 y	4.5 ± 0.2	97.6 ± 0.8	5.5 ± 0.3	20.8 ± 1.4	27.8 ± 3.3
F 14–18 y	4.2 ± 0.2	97.3 ± 0.9	5.5 ± 0.3	24.0 ± 1.5	21.9 ± 2.6
F 19–30 y	3.8 ± 0.2	98.3 ± 0.6	5.2 ± 0.4	27.7 ± 1.7	14.2 ± 1.7
F 31–50 y	3.6 ± 0.2	98.6 ± 0.6	4.8 ± 0.3	31.3 ± 1.9	12.0 ± 1.8
F 51–70 y	3.3 ± 0.1	98.6 ± 0.5	4.4 ± 0.3	36.2 ± 2.1	8.2 ± 1.4
F ≥ 71 y	2.9 ± 0.2	99.2 ± 0.4	4.0 ± 0.2	41.2 ± 2.5	5.4 ± 1.2
M 9–13 y	5.0 ± 0.2	94.6 ± 1.3	6.3 ± 0.4	19.2 ± 1.3	31.6 ± 3.1
M 14–18 y	5.5 ± 0.2	90.6 ± 1.6	7.6 ± 0.5	24.5 ± 1.7	21.2 ± 2.8
M 19–30 y	5.3 ± 0.2	92.5 ± 1.6	7.7 ± 0.7	28.3 ± 2.0	13.0 ± 2.4
M 31–50 y	4.8 ± 0.2	93.8 ± 1.3	5.9 ± 0.4	31.1 ± 1.9	13.4 ± 2.1
M 51–70 y	4.2 ± 0.2	95.7 ± 1.1	5.6 ± 0.4	35.6 ± 2.1	8.3 ± 1.6
M ≥ 71 y	3.6 ± 0.2	98.0 ± 0.7	4.7 ± 0.3	39.9 ± 2.4	6.2 ± 1.2
All ages	4.2 ± 0.2	96.5 ± 0.8	5.1 ± 0.3	31.5 ± 1.8	14.1 ± 1.8

<sup>a</sup>Prevalence of inadequacy is measured as % below the EAR (estimated average requirement) as the proportion of the population that does not meet requirement (IOM, 2011).

F, female; M, male.

Data are from Vatanparast et al., 2020 [15].

**FIGURE 57.1** Prevalence of supplement use of Canadians by age and gender in two national surveys: 2004 (prior to Health Canada having a vitamin D recommendation) and 2015 (after the 2007 Food Guide having a supplement recommendation). \* $P < .05$  between surveys. M, male; F, female. Source: Vatanparast et al., 2020 [15]



**FIGURE 57.2** The percentage of Canadians aged 6–79 y with plasma 25(OH)D below 50 nmol/L in the 2007–09 Canada’s Community Health Survey. Proportion of the Canadian population (age 6–79 y) with low 25(OH)D [ $<50$  nmol/L] with or without use of vitamin D-containing supplements in winter (W) and summer (S), and by self-reported nonwhite (NWh) ethnicity. Source: Whiting *et al.*, 2011 [19]

and 20  $\mu\text{g}/\text{d}$  (800 IU), respectively, were introduced by the IOM/Health Canada in 2011 [7]. These 2011 RDAs make the current supplement recommendation inadequate for those over 70 years. Health Canada has announced measures to improve fortification of staple foods in which amounts will be doubled for margarine and cow milk, with further provisions to allow more voluntary fortification with vitamin D. These new measures are slated for 2023.

Government guidance on vitamin D supplementation needs to stay informed about changes in dietary intake recommendations as insufficient doses can place much of the population at risk of not meeting the RDA, leading to inability to maintain adequate vitamin D status over winter. Promotion of vitamin D supplements for older adults likely stemmed from organizations such as Osteoporosis Canada ([www.Osteoporosis.ca](http://www.Osteoporosis.ca)), which may account for their higher use in older adults in 2004 that persisted in 2015. The lowest use of vitamin D-containing supplements was seen in males and females aged 4–18 years who typically are not concerned about osteoporosis.

Foremost in deciding the need for government advice on vitamin D supplementation of the population, a country should question whether there is substantial need since adequate sun exposure can have a large enough impact to mitigate deficiency due to a winter decline and/or other factors that block UVB exposure. For countries such as Canada, the best time to determine this is using survey data gathered during winter when most of the population is experiencing a lack of UVB as “vitamin D winter.” To this end, there is evidence that the 25(OH)D concentration of Canadians in winter is affected by supplement use [19]. Fig. 57.2 shows that for users of vitamin D supplements, the proportion of Canadians with circulating 25(OH)D less than 50 nmol/L ( $<20$  ng/mL) are well below that of nonusers in both winter and summer.

Of those who did not use supplements in winter or summer, over 50% had 25(OH)D levels  $<50$  nmol/L, while for those using supplements, only 17% in summer and 25% in winter were below 50 nmol/L. It should be noted that one-quarter of supplement users used a dose that was less than 10  $\mu\text{g}/\text{d}$  (400 IU), which may explain why some supplement users did not maintain adequate status during the entire survey collection period [20]. At the time of this survey collection (2007–09), the old recommendations of 1997 [18] were in use.

For Canadians who self-reported as an ethnicity with typically darker skin (i.e., who would be considered as nonwhite), those using vitamin D-containing supplements had half the prevalence of low 25(OH)D in winter (Fig. 57.2) [19] than those Canadians who did not use a supplement. Thus, it is apparent that users of vitamin D-containing supplements have better vitamin D status across race/ethnicity groups, despite differences in sun-blocking skin pigmentation. More importantly, those having darker skin color living in a country with a significant “vitamin D winter” should be encouraged to use supplements because the level of vitamin D fortification in Canada during the 2007–09 survey was not adequate. The recent change in supplement recommendations from Health Canada described in Table 57.4 also includes a statement that “many factors affect the amount of vitamin D the body makes from sunlight”; skin synthesis cannot be relied upon, and therefore, “a daily dietary source of vitamin D is recommended.”

Using data from nationally representative NHANES surveys in the United States, it appears that race/ethnicity can influence the use of supplements, which in turn affects vitamin D prevalence of inadequacy [21]. Table 57.6 shows vitamin D intakes for two darker skinned race/ethnic populations in the United States compared with whites (referred to as non-Hispanic White): non-Hispanic Black and Hispanic. For the two

**TABLE 57.6** Intake of vitamin D (food including beverages, fortified foods, supplements) by race/ethnicity, NHANES 2007–10.

	Non-Hispanic White	Non-Hispanic Black	Hispanic
<b>Vitamin D Intake (µg/d)</b>			
Naturally occurring	1.6	1.7	1.7
+ Fortified	5.4	4.5	5.7
+ Supplemented	14.2	9.5	8.3
% inadequacy			
Naturally occurring	100	100	100
+ Fortified	93	97	91
+ Supplemented	65	80	79

NHANES, National Health and Nutrition Examination Survey of the United States.

Data are from Malek et al., 2019 [21].

minority groups, intake of vitamin D from supplements only is about 66% of that of the non-Hispanic White population; nonetheless, all groups taking vitamin D supplements markedly lowered the percentage with inadequate intakes. Demographic factors that influence the use of vitamin D-containing supplements in different populations are described in the next section and may relate to education, income and other factors that lead to health inequities.

## 5. Characteristics of vitamin D supplement users

National nutrition surveys can capture the demographic and socioeconomic characteristics of supplement users to determine whether supplement use can be an indicator of potential for health inequities. Table 57.7 presents the characteristics of Canadian supplement users along with adjusted odds ratios (ORs) that provide the likelihood of those in the Canadian population having used or not having used a vitamin D supplement [20]. Those ORs greater than 1 indicate greater likelihood of D supplement use. Adjustment was made for season, age, income, chronic diseases, and milk consumption. Age was not a consistent factor between males and females except for adults over 40 years of age, indicating both male and female older adults (>60 years) were much more likely to use supplements than younger groups. Being female was not consistently associated with supplement use. While older women were twice as likely to take vitamin D supplements as older men, this difference was not significant.

**TABLE 57.7** Likelihood of taking a supplement containing vitamin D (N = 5604) using nationally representative data from the Canadian Health Measures Survey cycle 1 (2007–09).

Characteristic	Adjusted odds ratio (95% confidence Interval)	
	Females	Males
<b>Age (y)</b>		
6–11	1.15 (0.67, 1.97)	1.77 <sup>a</sup> (1.19, 2.64)
12–19	0.63 <sup>a</sup> (0.41, 0.96)	0.58 (0.33, 1.02)
20–39	1.0 (Reference)	1.0 (Reference)
40–59	1.56 <sup>a</sup> (1.10, 2.20)	1.46 (0.77, 2.79)
60–79	4.19 <sup>a</sup> (2.69, 6.54)	2.41 <sup>a</sup> (1.54, 3.77)
<b>Income</b>		
Higher	2.29 <sup>a</sup> (1.26, 4.16)	2.12 <sup>a</sup> (1.49, 3.00)
Middle	1.59 <sup>a</sup> (1.03, 2.46)	1.55 <sup>a</sup> (1.04, 2.31)
Lower	1.0 (Reference)	1.0 (Reference)
<b>Chronic disease</b>		
At least one	1.48 <sup>a</sup> (1.06, 2.06)	1.29 (0.76, 2.18)
None	1.0 (Reference)	1.0 (Reference)

<sup>a</sup>Significant (CI does not overlap with reference group). Adjusted for season, age, income, chronic diseases, and milk consumption (females only). ORs greater than 1 indicate greater likelihood of D supplement use compared with the reference group. Reference [20].

Income was an important factor for supplement use. Using the lowest income group as the reference, those in middle- and higher-income groups were significantly more likely to be a user of a vitamin D supplement. This finding supports the “inverse supplement hypothesis” [22], which states that people at risk for inadequacy (and who also might need a greater amount of a nutrient because of disease risk) are not the ones who take supplements. Poverty and food insecurity could create a situation of poor nutritional status. Finally, females but not males, who reported having one or more diagnosed chronic disease such as cancer, hypertension, heart disease, or osteoporosis, were 50% more likely to take a vitamin D supplement. This is not unexpected as vitamin D is commonly included in calcium supplements, and both nutrients were recommended for osteoporosis prevention by groups such as Osteoporosis Canada [23].

Asking detailed questions about attitudes and perceptions of vitamin supplement use can be difficult in national surveys as these are time-consuming. A small survey from the United Kingdom published results in which a questionnaire was used to gather such information [24]. Shown in Table 57.8, the reasons for not taking a vitamin D supplement are outlined, with response

**TABLE 57.8** Reasons for not taking a vitamin D supplement: Respondents in a small study (n = 209) of adults in the United Kingdom.

Answers	% answers In affirmative
<b>Researchers predetermined responses</b>	
"I think I get enough"	36
Unaware of benefit of taking them/lack of research on benefit	26
"I don't know which one I should take"	22
I don't think it's important	9
Too expensive	4
I don't know how to get them	3
<b>Other participant answers</b>	
Forgetful/lazy/busy	10
Unnatural/dislike taking tablets	3

Reference [24].

prevalence noted. The answer given by one-third of participants was "I think I get enough," while one-quarter of the participants were not aware of any benefit. Taken all together including the reply "I don't think it's important" covers more than two-thirds of the responses failing to acknowledge the importance of adequate vitamin D intake. Expense was not important; however, the participants were well educated, thus suggesting they may have a higher-than-average income. Being busy or forgetful was answered by 10%. These findings suggest that education and awareness might be important aspects to consider by governments making recommendations on vitamin D supplement use for the general healthy public.

The participants from the O'Connor study [24] (Table 57.8) were highly educated and had volunteered for this survey, whereas, obtaining information from low income, less motivated participants is much more difficult. We have conducted focus groups with low-income Canadians who were compensated for their time, to secure information about healthy eating and supplement use in general [25]. We determined that there are five general barriers to healthful eating and to the use of vitamin/mineral supplements: knowledge, income, accessibility, health, and preferences. There were two concerns expressed by participants relevant to why vitamin D supplements might not be used by low-income groups. First, participants said that the cost was a factor in that supplements must be purchased all at once and that a daily estimated cost of \$0.10, while low, meant that money that would have gone for food or living expenses was diverted to a bottle of supplements that can range in cost up to \$20 - \$40 depending on size. Second, participants were not encouraged to take supplements by any authoritative source such as a health-care professional or government agency, and no effort was made to explain how supplements might be used to fill critical dietary gaps [25].

## 6. Considerations when choosing to take a vitamin D supplement

As was shown in Table 57.2, some governments have made supplement recommendations for the population. However, to say "take a supplement" now puts the consumer in the position of choosing the right supplement. What is listed in Box 57.1 are the many considerations that appear online in articles or advertisements directed at the public. Some are straightforward, and evidence is clear while for others there may be a lack of consensus

### BOX 57.1

#### Decisions consumers need to make when choosing a vitamin D supplement regimen

- Vitamin D<sub>2</sub> or D<sub>3</sub>
- Source of D<sub>3</sub>  
*Fish liver oil, lanolin, algal, lichen, synthetic*
- Daily or bolus
- Coingestion with a lipid source
- Coingestion with other micronutrients  
*Calcium, vitamin K, magnesium*
- Product quality



on key issues. Not shown here are cost, physical form of the supplement (tablet, liquid, gummy, spray, soft gel, chewable), and dose.

### 6.1 Type of vitamin D: D<sub>3</sub> or D<sub>2</sub>

There is historical as well as recent evidence that vitamin D<sub>2</sub> has similar activity and general functions in bone as vitamin D<sub>3</sub>, i.e., the form we synthesize in our skin with adequate UVB exposure. Recent research findings now point to concerns that vitamin D<sub>2</sub> does not replicate vitamin D<sub>3</sub> in all activities; thus, many believe D<sub>2</sub> supplements are less effective in promoting optimal health. The frequency of dosing is also different for D<sub>2</sub> and D<sub>3</sub> as shown in acute studies comparing the efficacy of vitamins D<sub>2</sub> and D<sub>3</sub> in raising serum 25(OH)D concentration. Vitamin D<sub>2</sub> is less effective than D<sub>3</sub> when given as a single bolus [26]. Over longer time periods, some studies show higher efficacy of D<sub>3</sub>, while others show equal efficacy [27]. A recent longitudinal analysis comparing vitamins D<sub>2</sub> and D<sub>3</sub> found they do not have similar effects on the human immune system [27] for which vitamin D supplementation has gained more attention because of the COVID-19 pandemic. Furthermore, availability of vitamin D<sub>2</sub> as an over-the-counter (OTC) supplement is often limited. For consumers purchasing vitamin D supplements, in many countries such as Canada, there are few vitamin D<sub>2</sub>-containing choices, underscoring the public preference for D<sub>3</sub> supplements. One large Canadian supplement manufacturer offers 21 products listed on its website, and none contained vitamin D<sub>2</sub>. Nevertheless, with online cross-border shopping, it is possible to obtain vitamin D<sub>2</sub>. Historically, the reason for continued individual preference for D<sub>2</sub> supplements and food fortification was understood to be avoidance of animal-derived products by vegans, vegetarians, and several cultural and religious groups. This reasoning may no longer hold since now there are vegan options for vitamin D<sub>3</sub> supplements, but not fortification other than the traditional use of D<sub>2</sub> from vegetative sources. Despite the continued controversy over vitamin D<sub>2</sub> efficacy, the fact remains that vitamin D<sub>2</sub> supplements and vitamin D<sub>2</sub> use in fortification are better than no supplementation or food fortification.

### 6.2 Sources of vitamin D<sub>3</sub>

The earliest vitamin D supplement was fish liver oil, and even today, this is often the choice of many who want to obtain both vitamin A and D, or for whom this is a traditional product. This product is listed in Table 57.7, which provides a list of various vitamin D-containing supplements. Fish liver oils are extracted

from either cod or halibut. The problem with fish liver oils is to their required extensive cleaning of these products and the need to reintroduce fat-soluble vitamins at levels not found in nature. Thus, they often contain low amounts of vitamin D along with high amounts of vitamin A as retinyl palmitate. For example, in Canada, some products have only 200 IU/dose (5 µg) of vitamin D yet may contain close to the upper level (3000 µg) of retinol. Retinol has the potential to interfere with vitamin D metabolism [28] although not enough research has been done to verify this relationship.

Most vitamin D<sub>3</sub> supplements are derived from lanolin, which is sourced from sheepskin and wool. In the process of cleaning wool, lanolin is extracted to be saponified, washed, and centrifuged to provide pure crystalline 7-dehydrocholesterol that is subsequently illuminated with UVB to form cholecalciferol [29]. Information of these processes is found at various company websites. Until recently, there were no concerns about using lanolin as the source of vitamin D in supplements except for vegetarians and vegans who would look for a yeast-derived source, which was vitamin D<sub>2</sub>. However, with vitamin D<sub>2</sub> no longer being considered as an equivalent to vitamin D<sub>3</sub>, many manufacturers have sought out plant- or algal-based vitamin D<sub>3</sub> sources.

There are now several vegan sources of vitamin D<sub>3</sub>: algal and lichen. Microalgae such as *Nannochloropsis oceanica* are able to produce vitamin D<sub>3</sub> when irradiated with UVB [30]. Algal supplements of vitamin D, however, are not widely available. There is at least one major company producing vitamin D<sub>3</sub> supplements using lichen. Lichens constitute a symbiotic association between a fungus and algae and/or cyanobacteria. The lichens grow in sparse clumps in northern regions like the Canadian tundra, but lichens used for supplements are farmed outdoors where vitamin D<sub>3</sub> is produced naturally.

### 6.3 Coingestion with a lipid source

As vitamin D is a fat-soluble vitamin, it would seem appropriate to recommend taking supplemental vitamin D with a lipid source; but not all studies show this to be necessary. In a study of the effectiveness of fortification, similar doses of vitamin D<sub>2</sub> were compared after adding to whole (full fat) milk, skim milk, corn oil on toast and ingested by adult subjects. Despite differences in fat content, there were no differences in absorption (measured as appearance of the parent molecule in the subsequent 72 h) [31]. The coingestion of vitamin D and lipid may be relevant to food fortification but not to OTC vitamin D<sub>3</sub> supplements based on current manufacturing sources. All vitamin D supplements sold have fat included in the tablet or drop, such as flaxseed or other plant oils,

or refined lipids such as magnesium stearate. The question has arisen whether one should consume a supplement with foods as this is commonly recommended for many nutrient-based supplements. In a systematic review of factors affecting vitamin D absorption, it was concluded, but using only weak evidence, that vitamin D was better absorbed when it was consumed with fat-containing meals and that absorption would occur without fat or oily vehicles [32].

## 6.4 Coingestion with another micronutrient

For several decades, calcium supplements have been sold with vitamin D as an additional active ingredient, to provide these two nutrients for prevention or treatment of osteoporosis. Ingestion at the same time is not required as it is not the parent compound, cholecalciferol, which stimulates calcium absorption but the active metabolite, 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ). Manufacturing supplements with calcium and vitamin D together was for consumer convenience.

Recently, vitamin D supplements containing vitamin K have appeared in the marketplace. Similar to the situation with calcium, the addition of vitamin K is for convenience. There is limited but biologically plausible evidence that vitamin K may mitigate vascular calcification and thus reduce the chances of excess vitamin D causing hypercalcemia. This is accomplished by Matrix Gla Protein (MGP), which is a small Gla vitamin K-dependent protein that acts as an inhibitor of calcification [33]. Often the form recommended is vitamin K<sub>2</sub>, which is found in fermented food but also can be made in vivo from the plant form of vitamin K, which is K<sub>1</sub> [34]. As intakes of vitamin D in a physiological range do not have risk of hypercalcemia, this additional vitamin K for the purposes of mitigating hypercalcemia is not essential. Future vitamin K recommendations will likely emerge for prevention and treatment of osteoporosis; thus, inclusion of vitamin K in a D supplement will facilitate taking two bone healthy nutrients [34]. Until recently in Canada, having vitamin K included in OTC supplements was not allowed due to risk of vitamin K promoting clot formation in those taking certain anticoagulants such as warfarin, but this restriction has been rescinded, in part due to availability of anticoagulants that are not vitamin K inhibitors.

Magnesium's role in vitamin D metabolism is actively being researched. Magnesium is emerging as being necessary in the biosynthesis, transport, and activation of vitamin D to its metabolites. With magnesium deficiency, there is impairment of parathyroid hormone (PTH), which in turn prevents renal  $1\alpha$ -hydroxylase-mediated synthesis of  $1,25(\text{OH})_2\text{D}$  [35]. One can purchase a supplement containing vitamin D and

magnesium together; it is usually recommended that magnesium supplements also be taken. Low magnesium intakes can be rectified through eating a healthy diet, for example, Canada's new Food Guide that recommends that half one's plate be filled with fruits and vegetables, provides enough magnesium to meet current recommendations yet is low in vitamin D [36]. In this case, a consumer would be advised to get magnesium from food and take a vitamin D supplement to fill the dietary gap.

## 6.5 Taking vitamin D supplements daily versus bolus dosing

The question arises about how often a vitamin D supplement should be taken, i.e., daily, weekly, monthly, or quarterly. All the government recommendations in Table 57.2 specify "daily." Yet it is well known in the pharmaceutical world that taking drugs less often improves adherence, and it is not uncommon to find medications such as oral bisphosphonates available in daily, weekly, or monthly formulations, with monthly having the highest compliance and persistence [37]. Vitamin D supplementations differ in that there is consensus that daily is the best course [38]. This is not to say that bolus dosing as a loading dose should be questioned, as this addresses a different function. This chapter is concerned with supplementation that maintains vitamin D status over time, not the issue of repletion—the latter circumstance responding well to bolus dosing. Further, the body needs all the metabolites of vitamin D, including the parent molecule [39]. The longest-lived metabolite is  $25(\text{OH})\text{D}$ , which has a half-life of about 2–3 weeks in the circulation and around 2–3 months for the whole body; in contrast, vitamin D has a half-life of roughly 24 h and the active form  $1,25(\text{OH})_2\text{D}$  is less than 12 h in the circulation [40]. The parent compound cholecalciferol should be available on a daily basis to ensure stable circulating concentrations.

## 6.6 Product quality

Globally the supplement industry has not always earned a good reputation for its supplement practices including making false claims for curing disease [41]. Different countries handle this industry in different ways. In Canada, nutrient-based supplements must have a natural product number (NPN) on the bottle. In the management of natural health products (NHPs) in the Canadian market, there is a strategy to address the most common issues such as contamination, adulteration, and deceptive or misleading advertising practices by regulating NHPs along with other health products available without a prescription (i.e., "over the counter,"

**TABLE 57.9** Vitamin D containing supplements in Canada available over-the-counter (OTC) in pharmacies and health food stores.

Brand Name or type of product	Vitamin D <sub>3</sub> Dosage on label <sup>a</sup>
Vitamin D supplement	400, 600, 1000, 2500 <sup>b</sup> IU
Multivitamin	400–1000 IU
Specific supplements, e.g., probiotic, immune	400–1000
Calcium supplement with vitamin D	50–400 IU
Vitamin D supplement with one other nutrient such as vitamin K or vitamin A	400, 500, 800, 1000 IU
Fish liver oil	200, 400 IU

<sup>a</sup>Amounts shown do not include overage amount that can be 20%.

<sup>b</sup>See Box 57.2 for warning label on this product.

Sources: Company websites.

OTC) in a single federal division [42]. The “NPN” is similar to a “DIN,” which is the drug identification number. Many supplements sold worldwide may have the letters USP (U.S. Pharmacopeia), which indicates this particular supplement has been tested for safety and quality by this non-Federal group working in alliance with the US Food and Drug Administration to ensure product safety.

## 7. Safety of vitamin D supplements

Table 57.9 shows a list of the variety of vitamin D supplements that are available for purchase without prescription from pharmacies and from stores selling natural food products in 2022 in Canada. When

examined by dose, supplement users in Canada whose data are plotted in Fig. 57.2 had a choice of using one or more products as a source of vitamin D. These survey data were collected in 2007–09, and at that time, a relatively small percentage of supplement users (16%) took a total dose >25 µg (1000 IU). Only recently has a product with a dose greater than 25 µg (1000 IU) been allowed to be sold, and it carries a very lengthy warning label created by Health Canada that is described in the following section addressing safety concerns (see Box 57.2). Consumers may also shop online, and this gives access to higher dosage levels available from other countries such as the United States where doses as high as 5000 IU (125 µg) may be sold OTC without prescription.

The dietary reference intakes included for the first time in 1997 an upper level (UL) of safe intake for vitamin D, also referred to as an upper limit in other countries [7,18,43]. These are shown in Table 57.10 and indicate near agreement in setting ULs between Canada/United Kingdom and Europe/United Kingdom. The ULs are primarily concerned with risk of adverse effects when supplement intakes, sometimes alone as in the case of folic acid, or in combination with food-derived intakes as is the case for vitamin D, approach levels that show measurable harm. The UL value itself, however, is considered safe for consumption and marks the beginning of increasing risk for adverse effects. In almost every case for a micronutrient (the exception being the current 1997 UL for magnesium) [18], the UL is considered to be chronic intake over a long period of time, not acute intake of a very high dose leading to immediate toxicity.

High intakes following self-dosing with excessive amounts of vitamin D supplements have been

### BOX 57.2

#### Warning label attached to a bottle of vitamin D supplements with dosage of 2500 IU (67.2 µg) introduced in 2022 to be permitted to be sold in Canada in pharmacies and health food stores.

Warning: keep out of reach of children and pets. 15–20 mcg (600–800 IU) of vitamin D per day is adequate for most individuals. Consult a healthcare practitioner to determine if you would benefit from additional vitamin D before taking this product. Do not use this product if you have hypercalcemia and/or hypercalciuria. Consult a healthcare practitioner prior to use if you are pregnant or breastfeeding; have a kidney disorder; take other vitamin D supplements, multivitamin supplements containing vitamin D, or products containing vitamin D analogs; take any recommendation medications including antacids, anticonvulsants, digoxin, cholestyramine, colestipol, mineral oil, steroids, statins, or thiazide diuretics. Stop use and consult a healthcare practitioner if weakness, fatigue, drowsiness, headache, lack of appetite, dry mouth, metallic taste, nausea, vomiting, vertigo, ringing in the ears, lack of coordination, and muscle weakness occur, which are early symptoms of hypercalcemia, or if you have any other side effects.

**TABLE 57.10** Comparison of safety assessments for vitamin D for healthy populations: Canada and United States (IOM), Europe (EFSA) and United Kingdom (SACN).

Country/ organization	Age	Upper level µg (IU)/day	Criterion for risk
Canada and United States (institute of medicine, IOM) 2011	0–0.5y	25 (1000)	Maintenance of a serum 25(OH)D level below 150 nmol/L of 125 µg (5000 IU)/day, and dividing by 1.25 UF
	0.5–1y	37.5 (1500)	
	1–3y	62.5 (2500)	
	4–8y	75 (3000)	
	9+ y	100 (4000)	
Europe (EFSA) 2012 and the United Kingdom (SACN) 2016	0–1y	25 (1000)	Risk of hypercalcemia UL set using NOAEL of 250 µg/day (10,000 IU/d), and dividing by 2.5 UF
	1–10y	50 (2000)	
	≥11y	100 (4000)	

NOAEL, no-observable-adverse-effect level; UF, uncertainly factor.

Sources: Institute of Medicine 2011 [7], European Food Safety Authority (EFSA) 2012 [43], Scientific Advisory Committee on Nutrition (SACN) 2016 [9].

described, although the dose required to induce vitamin D toxicity is uncertain [44]. The major identifiable risk for vitamin D excess is hypercalcemia, which promotes calcification and thus damage of soft tissues. Vitamin D intoxication does not arise from the consumption of conventional foods (including fortified foods) or by excess exposure to UVB [45]. Cases have arisen, although, from accidental overfortification of milk with vitamin D<sub>3</sub>, from uncontrolled use of vitamin D mega doses, and from inappropriate use of vitamin D metabolites. Clinical signs of vitamin D toxicity include hypertension, hypercalcemia, hypercalciuria, and extraosseous calcification [45]. These clinical symptoms may arise after serum 25(OH)D levels exceed 250 nmol/L for an extended period of time.

The ULs are set for chronic exposure by the healthy population, not for those who may have genetic or other factors, which may predispose them to sensitivity to a nutrient. For example, those infants with the genetic disorder idiopathic infantile hypercalcemia experience symptoms of vitamin D toxicity at intakes no more than 500 IU [46]; the cause of this is mutations in gene for *CYP24A1*, which encodes the 24-hydroxylase enzyme responsible for inactivating vitamin D. These children are unable to metabolize vitamin D metabolites to inactive compounds to be excreted. Other causes of infantile hypercalcemia are being investigated [47]. Detailed information of this disorder is provided in Chapter 71.

In Table 57.10, ULs for vitamin D set in 2011 by the IOM [7] apply to Canada and the United States and have been adopted by many countries around the world. As is usual with setting ULs, a no-observable-adverse-effect level (NOAEL) was chosen based on

published data, which was a 5-month study using various doses of vitamin D [48] during which no adverse effects such as hypercalcemia or hypercalciuria were reported. The IOM panel used vitamin D intake of 5000 IU/day, which achieved serum 25(OH)D levels that ranged between 100 and 150 nmol/L and applied an uncertainty factor (UF) to bring this intake to 4000 IU (100 µg) per day. ULs for children under 9 years were adjusted downward to account for potential greater risk of high intakes. The European Food Safety Authority (EFSA) and the United Kingdom (SACN report) are similar to the 2011 IOM values, with small differences in ULs for children as well as cutoffs for ages [9,43].

ULs are used by governments to regulate the doses allowed in supplements as well as the extent of fortification in terms of the number of products and amount of vitamin D per serving. Governments can also use ULs to provide information to the public. In the case of Canada, doses of vitamin D allowed to be sold were restricted to 1000 IU (25 µg) per unit until 2021 when a product with 2500 IU (62.5 µg) was permitted to be sold (Table 57.9). The product having this allowable dosage has a very extensive Health Canada warning label affixed to the bottle (Box 57.2). This warning label describes vitamin D toxicity symptoms in both English and French as a safety precaution alerting the consumer. The label and warning insert does not use the term “UL.” It is likely that the permitted dose of 2500 IU was chosen as it is the UL for children aged 1–3 years and is under the UL specific to those age 4 and older (Table 57.10).

Since setting the UL for vitamin D, several other long-term studies of large doses of vitamin D have been published. A study of 4000 IU (100 µg) given to adults at risk for diabetes for 2.5 years reported no adverse effects from using this regimen [49]. Contrary to these earlier findings of safe use of high doses, a second randomized controlled trial (RCT) of high daily doses of vitamin D<sub>3</sub> (10,000 IU [250 µg]) and 4000 IU (100 µg) compared with 400 IU (10 µg) given over 3 years has provided new data on potential risk of high intakes of vitamin D on bone mineral density (BMD) in adults over 50 years of age [50,51].

Table 57.11 presents the results from this trial of 400 IU, 4000 IU, and 10,000 IU over 3 years in adults over 50 years of age. Achieved levels of serum 25(OH)D, the primary outcome that was the measurement of volumetric BMD, and adverse effects reported for this RCT are shown in Table 57.11 [50,51]. The group receiving 400 IU (10 µg) is considered the control group as their total intake of vitamin D from food and supplement was 600 IU (15 µg), which is the current adult RDA in Canada. Of note, baseline 25(OH)D levels for all participants living far north in Calgary averaged close to 75 nmol/L, an unusually high baseline for Calgary residents. The group receiving the lowest dose equivalent to less than



**TABLE 57.11** Safety and efficacy of three doses of vitamin D supplementation (400 IU, 1000 IU, 10,000 IU) for three years in adults aged 55–70 y living in Calgary.

Treatment group	+ 400 IU/d (10 µg) N = 124	+ 4000 IU/d (100 µg) N = 125	+ 10,000 IU/d (250 µg) <sup>a</sup> N = 124
25(OH)D nmol/L <sup>b,c</sup>			
Baseline	76.3	81.3	78.4
3-month	76.7	115.3	188.0
3-year	77.4	132.2	144.4
3-Year change in volumetric bone mineral density <sup>b</sup>			
Radial	−1.2	−2.4	−3.5
Tibial	−0.4	−1.0	−1.7
Hypercalciuria <sup>d</sup>			
Events over 3 y	27	47	56*
Hypercalcemia <sup>d</sup>			
Events over 3 y	0	4 <sup>e</sup>	12 <sup>e*</sup>
All clinical adverse events <sup>d</sup>			
Events over 3 y	836	920	824

<sup>a</sup>This dose was inadvertently reduced to < 250 µg/d between months 18–36 due to product degradation.

<sup>b</sup>Reference [50].

<sup>c</sup>Background dietary vitamin D was 5 µg/d.

<sup>d</sup>Reference [52].

<sup>e</sup>All episodes of hypercalcemia were mild (2.56–2.64 mmol/L) and resolved on follow-up testing with recommendation to reduce calcium supplementation during the trial.

\*Significantly different from lowest dose group.

the RDA (400 IU) had no change from this high value over the 3-year period. At 3 months and 3 years, those receiving the UL (4000 IU [250 µg] per day) had serum 25(OH)D levels below 150 nmol/L (on average). Shown in the authors' published box and whisper plots, one can discern that circulating 25(OH)D was below 150 nmol/L for 75% of the group throughout the trial [51]. Of concern, the highest dose did put participants well over 150 nmol/L, with a 3-month average level at 188 nmol/L. By the 3-year end of the trial, the average had fallen to 144 nmol/L. The observed decline at 3 years in the 10,000 IU group had a problem with varying degrees of degradation of vitamin D at 18 and 36 months. The box and whisper plots at the 18-month timepoint showed 25% of these participants had 25(OH)D concentrations well over 200 nmol/L. Table 57.11 shows the primary outcome of BMD was not improved with additional vitamin D (over 15 µg, the RDA based on bone health), and there is indication of greater loss of volumetric BMD by women given the

two higher doses. There was transient hypercalciuria and hypercalcemia in the two higher dose groups that were resolved with reduction of dietary calcium. There were no observable clinical adverse event differences among the three groups.

## 8. Guidance is needed for use of vitamin D supplements

The UL for vitamin D was set to provide the public with an intake that should not be exceeded unless the person is under the care of a health provider. Several of the vitamin D supplement recommendations shown in Table 57.2 indicate the need to consult a physician or other healthcare provider when considering a supplement, and it may be that the general public likely does not know that ULs exist or what adverse effects could occur with high intakes of a fat-soluble micronutrient. To illustrate this lack of knowledge, we reanalyzed a study from the United States where vitamin D supplement use by seniors living in an assisted care nursing home was described [53]. Table 57.12 shows the residents' use of vitamin D supplements (as either D<sub>2</sub> or D<sub>3</sub>) by dosage amount compared with achieved 25(OH)D. Taking 100 µg (4000 IU) or less kept 25(OH)D measures below that of 150 nmol/L, but when intakes were above this amount, which is the UL, some of the residents were above 150 nmol/L. How the residents took vitamin D was described: as vitamin D alone (which in the United States could be as doses as high as 5000 IU), in calcium supplements, in multivitamins, or as a combination of more than one of those three options. The most effective supplements for achieving a serum 25(OH)D of 75 nmol/L were the D-alone and the combination, and the authors calculated that 37.5 µg (1500 IU) was sufficient for this purpose. In examining the data, we noted that people without

**TABLE 57.12** Use of vitamin D supplements by residents (>65 years) of nursing homes in Texas, United States (N = 173) who made personal choices for supplement use.

Supplement Dose	N (%)	Achieved mean 25(OH)D nmol/L
0 (none used) (Diet = 5 µg/d)	70 (40%)	60
10–20 µg/d (400–799 IU)	27 (16%)	73
20–50 µg/d (800–1999 IU)	30 (17%)	88
50–100 µg/d (2000–3999 IU)	30 (17%)	104
>100 µg/d (>4000 IU)	16 (9%)	133

Reference [53].



**TABLE 57.13** Vitamin D calculators to estimate the need for supplemental intake for adults ranging from 50 nmol/L to 75 nmol/L at two different body weights: at normal weight (BMI = 25) or if obese (BMI = 30).

Name (country of origin)	Factors used in calculation	Recommendation to reach and maintain 75 nmol/L for adult of normal weight (70 kg)	Recommendation to reach and maintain 75 nmol/L for obese Adult (90 kg)
Omni Calculator (Poland) <sup>a</sup>	Age Height Weight Serum level (optional)	"Adults aged 18–50 years require at least 600 IU of vitamin D a day to maximize bone health and muscle function. The dose of at least 1500–2000 IU may be needed to raise the blood level of vit. D to consistently above 30 ng/mL"	"Obese adults should be given doses that are at least —two to three times greater than those given to meet their vitamin D requirements."
Vitamin D* Calculator (United States) <sup>b</sup>	Weight Serum level	"2000 IU (50 mcg) per day (this includes your current intake amount) will be sufficient for 50% of people to achieve the desired serum level of 75 nmol/L."	No difference from 70 kg
Omega quant Vitamin D calculator (United States) <sup>c</sup>	Weight Serum level	"Amount of vitamin D needed to reach your target blood level (including current intake): 1300 IU."	"Amount of vitamin D needed to reach your target blood level (including current intake): 1600 IU."

<sup>a</sup>Available at: [www.omnicalculator.com/health/vitamin-d](http://www.omnicalculator.com/health/vitamin-d).<sup>b</sup>Available at: [www.grassrootshealth.net/project/dcalculator/](http://www.grassrootshealth.net/project/dcalculator/).<sup>c</sup>Available at: <https://omegaquant.com/vitamin-d-calculator/#>

guidance could be at risk of taking too little or too much, and we recommended a dietitian or other healthcare professional be available to assist residents in their choice of supplementation [54]. As noted in Table 57.8, people express confusion about selecting supplements.

A comparison between providing vitamin D using enhanced food fortification and that of supplementation was recently made by the ODIN consortium of vitamin D researchers (see Chapter 56 for further details). They conducted a systematic review of safety issues arising from increasing vitamin D intakes through fortification and by supplementation [55]. In food-based or supplement trials involving over 3300 participants, where vitamin D doses ranged between 5 and 175 µg/d, there were no reported adverse effects. However, the prevalence of high serum 25(OH)D (>125 nmol/L) was <10% when vitamin D supplements were administered, and only <0.1% for fortified foods. As there are restrictions of the amount of vitamin D added to foods as opposed to amounts provided in supplements, no guidance is thought to be necessary for the former strategy other than choosing the food that is fortified.

While health professionals who are trained in nutrition would be the best source of information on supplement use, ways to provide guidance can be found online. A browser search of "vitamin D calculators" brings up several options for this purpose. Table 57.13 provides examples of three such calculators. Each one uses slightly different criteria, but all have been influenced by the van Groningen et al. [56] cholecalciferol loading dose guideline for vitamin D-deficient adults, indicating that the values provided are a combination

of repletion and maintenance amounts of vitamin D. The published formula is  $dose\ (IU)\ to\ achieve\ 75\ nmol/L = 40[75 - serum\ 25(OH)D] \times body\ weight\ (kg)$ . This formula was generated using patient data. It is important to note that body weight made a large difference in the amount of vitamin D recommended by these online calculators. Yet there is no consensus on the role body weight plays in vitamin D status. The current Institute of Medicine RDA for vitamin D is defined as the amount of vitamin D needed to maintain serum 25(OH)D at 50 nmol/L for almost everyone in the population [7]. Yet the value of 15 µg (600 IU) per day is the same for a 12-kg child of 1-year-old as a 70-kg adult man (highest reference weight). No other nutrient has the same RDA for children and adults, which for vitamin D does, up to age 70 years.

A further point on body weight is the effect of obesity on vitamin D status. The 2011 Endocrine Society's recommendations for vitamin D classify obesity as a factor that puts people in an at-risk group for vitamin D deficiency and hence increases their requirements. This has been borne out by research, but there is some uncertainty as to whether obesity causes vitamin D deficiency through sequestering cholecalciferol and 25(OH)D in adipocytes [40] or whether the behavior of those who are obese cause them to avoid skin baring, thus reducing UVB exposure. Some evidence for the latter is that this relationship is not always seen in both men and women [57] nor is it stable through all seasons [58].

Overall, there is a need for guidance for the public to navigate the many considerations that exist for vitamin D supplementation. The United States offers leadership

in this regard by having an Office of Dietary Supplements (ODS) within the National Institutes of Health. The ODS provides information to the public as well as to health professionals. The Fact Sheets that are directed to health professionals are a model of evidence-based information and are updated yearly. Links to the scientific literature as well as to resources make this a useful resource. The 2021 Fact Sheet for vitamin D contains over 170 references, and each has a link to its PubMed abstract [59]. The Fact Sheet for the consumer is in a Q&A style that addresses most commonly asked questions. Consumers have access to the more detailed health professional version.

## 9. Conclusion

We asked the question: “Is it possible to sustain vitamin D adequacy in the whole population with vitamin D supplementation?” We used Canada as a case study, as it is a country with at least five or more months of “vitamin D winter.” While Canada enforces mandatory vitamin D fortification of two staple foods, this is at a level that is only able to maintain an average intake of 5 µg/d (200 IU) per day. In recognition of this low level of vitamin D in the food supply, a recommendation for D-supplement use in adults over 50 years of age began in 2007 by the Ministry of Health. This recommendation has recently been updated to better coincide with RDAs that were set in 2011 [7]. Previously the supplement recommendation in Canada only addressed people over 50 years of age. Between 2004 and 2015, there was a modest increase in the prevalence of supplement use, from 28% to 33% [15]. For those supplement users, probability of inadequate intake was very low, and status of vitamin D in winter showed only one-quarter of the population below 50 nmol/L, while over half of those not using supplements had insufficient intakes and could not sustain adequate vitamin D status over winter. Supplementation is efficacious and at intakes below the UL is safe. There are, however, barriers to having supplement use, on its own, as a public health strategy. Those who take supplements have higher incomes and levels of education, whereas those lower income nonusers may not be able to afford foods with added or natural vitamin D that are in reality luxury foods, such as enriched eggs and wild-caught sockeye salmon, respectively. There are often a potentially confusing array of supplement choices and considerations that may leave people confused. One study showed people taking supplements took levels that were too low and too high, pointing to the need for guidance from health providers and sound educational resources from government.

With respect to a practical application for supplement use, countries with long vitamin D winters for various reasons experience high prevalence of inadequate intake and poor vitamin D status and have no effective public health policies in place to increase intake and sustain adequate vitamin D status over time. The combined policies of mandatory fortification and national guidelines and education for vitamin D supplementation currently shown to be somewhat effective in Canada may serve as a roadmap for developing appropriate strategies to augment intake and sustain optimal vitamin D status in most of the population over time. Both mandatory, enforced vitamin D fortification of commonly consumed food staples and government-supported education and advice concerning appropriately targeted and safe dosing regimens for D supplement use are critical components to achieving these goals.

## 10. Summary points

- Vitamin D supplement use reduces prevalence of inadequacy of vitamin D intake and decreases the proportion of people with insufficient status.
- Governments can make recommendations for vitamin D supplement use that will encourage the population in their use.
- There is health inequity between people with low income and high income with respect to supplement use and this needs to be addressed.
- Guidance from health providers and educational resources from government is needed so people know what to take and how much.
- Combined government policies of mandatory fortification of food staples and national guidelines and education for vitamin D supplementation will likely be the most effective population strategy for maintaining adequate vitamin D status and dietary intake.

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# Vitamin D and food fortification

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## OBJECTIVES

- To provide a brief overview of the prevalence of low vitamin D status in Europe, North America, and other regions around the globe.
- To consider the current deficits between vitamin D intakes and dietary recommendations to meet vitamin D requirements.
- To evaluate the multiple strands of evidence for a role for food fortification using vitamin D<sub>3</sub>, D<sub>2</sub>, and 25-hydroxyvitamin D, both in nutrient addition and biofortification modes, in preventing low vitamin D status in the population.
- To consider safety aspects of different strategies for increasing vitamin D in the food supply.
- To recommend a pathway to translate knowledge into policy development and implementation for prevention of vitamin D deficiency in the population.

## 1. Introduction

The role of vitamin D in the development and maintenance of bone health is well established [1], and there is a growing body of evidence for an association between low vitamin D status and increased risk of nonskeletal adverse health outcomes (including cardiovascular disease, hypertension, diabetes, certain cancers) (Ref. [2], see chapters on *Vitamin D and Type 2 diabetes, metabolic syndrome and hypertension* by A. Pittas, *Vitamin D and cardiovascular disease and hypertension* by S.

Pilz, and *Epidemiology of Vitamin D and cancer risk* by E. Giovannucci; in the current works) as well as immune defense and respiratory infection [3]. Notwithstanding the importance of ongoing discussions with respect to serum 25-hydroxyvitamin D [25(OH)D] concentrations that may increase the risk of ill health, there is widespread acknowledgment that low serum 25(OH)D is widespread in the community, affecting many millions of people, and that there is a pressing need to address the problem [4]. Taking a serum 25(OH)D concentration of 30 nmol/L as the cutoff below which the risk of clinical vitamin D deficiency increases (manifesting as nutritional rickets in children and osteomalacia in adults), the first priority from a public health perspective is to ensure that this risk is minimized [4].

### 1.1 Prevalence of low vitamin D status

Estimates of the prevalence of very low vitamin D status (based on standardized serum 25(OH)D < 30 nmol/L) in representative population samples in the United States (US) ( $n = 16,180$ ) [5], Canada [6] ( $n = 8351$ ) and Europe ( $n = 55,844$ ) [7] have been reported as 5.0%, 8.8%, and 13%, respectively. Worrisome as they are from a population perspective, these estimates do not highlight those at highest risk in the community, as they do not capture the differences in prevalence of low vitamin D status caused by factors such as age, sex, seasonality, geographical location, and ethnicity that place many groups at higher risk, which is often masked by higher 25(OH)D concentrations among the majority. For example, the prevalence of serum 25(OH)D < 30 nmol/L increases from 8.2% in summer to 17.7% in winter in Europe [7], and from 3.3% in summer to 9.3% in winter in the United States, based on the

2007–10 *National Health and Nutrition Examination Survey* (NHANES) [8], as the most recently reported cycle of the survey (2011–14) adjusted prevalence estimates for season from 2003 onward [5].

Across ethnic groups within NHANES 2011–14, the prevalence of serum 25(OH)D < 30 nmol/L in non-Hispanic white, Hispanic, non-Hispanic Asian, and non-Hispanic blacks in the US was 2.1%, 5.9%, 7.6%, and 17.5%, respectively [5]. The Canadian survey showed that the prevalence of serum 25(OH)D concentrations <30 nmol/L in nonwhite participants was over threefold higher than that of white participants (20.1% *v.* 5.9%, respectively) [6]. Adolescents and young adults are at higher risk than other population life stage groups: within NHANES 2011–14, which included children, the prevalence of serum 25(OH)D concentrations <30 nmol/L was 0.5% and 1.4%, respectively, in those aged 1–5 and 6–11 years and increased to ~5% in 12- to 19-year-olds [5]. In both the United States and Canadian surveys, the prevalence of serum 25(OH)D concentrations <30 nmol/L was highest among younger adults (typically ~8%–11% in those aged about 20–50 years) than older adults (~3%–4%) [5,6].

Largely attributed to clothing practices, it has been noted that women and girls, especially in low-income and middle eastern countries, have lower 25(OH)D than their male counterparts [9,10]. In a recent systematic review of 83 low- and middle-income countries [11], Afghanistan, Pakistan, India, Tunisia, Syria, the West Bank and Gaza, and Mongolia were classified as “hot spots” for very low vitamin D status among women, pregnant women or infants on the basis of having a prevalence of 25(OH)D (<25–30 nmol/L) above 20%. In Africa, Mogire et al. [12] reviewed 133 studies including 21,591 participants from 23 African countries. Women and newborn infants were at particular risk, especially in the north of Africa and urban areas of South Africa, with a prevalence of 25(OH)D < 30 nmol/L of 18.5%.

Despite widespread speculation around higher cut-offs for serum 25(OH)D for public health, there are increasingly harmonized, albeit not universal [13], proposals among a number of authoritative agencies for a serum 25(OH)D concentration of 50 nmol/L as a threshold likely to meet the requirements of almost all “normal healthy persons” ([14–17]; and see Table 58.1), equivalent to a personal, or clinical, target. The average yearly population prevalence of standardized serum 25(OH)D < 50 nmol/L in Europe, the United States, and Canada is 40.4%, 23.3%, and 36.8%, respectively [5–7]. The prevalence of 25(OH)D <30 and <50 nmol/L for other parts of the Globe, especially those which do not have substantial fortification of food, has been reviewed recently [18] and can also be seen in Chapter 54 in the current book.

## 1.2 Dietary recommendations to meet the status gap

Dietary recommendations for vitamin D, devised for population, as opposed to individual (or clinical) use, are calculated from dose–response studies of the response of serum 25(OH)D in winter to graded vitamin D supplementation doses. They vary largely based on the population goal of the agency responsible for developing them and the conceptual frameworks used by those agencies for setting dietary recommendations, which vary slightly. Put simply, the goal can be either for everyone in the population to achieve a minimum 25(OH)D target, such as in the United Kingdom, where each individual is recommended to take 10 µg/day vitamin D (population protective goal based on moving 97.5% > 25 nmol/L) [13], or for the average intake in the population to achieve 10 µg/day (also a population goal known as the estimated average requirement (EAR), based on getting 50% above a 25(OH)D threshold of 40 nmol/L) in the case of the United States [14]. Both the United States and the European Union [14,17] also recommended 15–20 µg/day on an individual basis to achieve personal targets of 50 nmol/L, and although these values are useful for individuals, they cannot be applied to population evaluations and are not applicable to this discussion. Detailed reviews of the dietary recommendations for vitamin D are available [19,20].

## 1.3 Sources of vitamin D and the role of food in meeting nutritional requirements

The major source of vitamin D in humans is sunshine; UVB radiation (290–315 nm) stimulates cutaneous synthesis of cholecalciferol, which is stored in adipose tissue or undergoes hydroxylation in the liver to 25(OH)D [14]. Environmental factors, such as latitude and prevailing weather conditions, determine whether sunshine of sufficient strength is available to stimulate the conversion of 7-dehydrocholesterol in the skin to precholecalciferol [21]. Personal attributes, such as skin pigmentation, age, attire, sunscreen, working environment, physical activity, and sun exposure behavior, can also prevent or impede vitamin D synthesis [21]. For further information on sunlight and vitamin D, readers are referred to Chapter 3 and Chapter 56 of the current book.

People resident at latitudes greater than around 40 degrees rely on body stores and vitamin D in the diet to maintain healthy vitamin D status all year round. Vitamin D occurs in food, both naturally and as a fortificant, as cholecalciferol (vitamin D<sub>3</sub>) and ergocalciferol (vitamin D<sub>2</sub>) and also in nutritional supplements. The well-described winter nadir in circulating 25(OH)D concentrations means that body stores, which are dependent on UVB exposure, are inadequate to maintain

**TABLE 58.1** Dietary Reference Values/Intakes for vitamin D from period 2011–16<sup>a</sup>.

DRV/DRI ...	UK RNI [13]		NORDEN RIs [16]	DACH AI [15]	EFSA AI [17]	North American [14] EAR RDA	
	≥25	≥50	≥50	≥50	≥40	≥50	
Serum 25(OH)D target (nmol/L)							
Age group				(µg/day) <sup>b</sup>			
0–6 months	8.5–10 <sup>c</sup>	10	10	—	10 <sup>d</sup>	—	
7–12 months	8.5–10 <sup>c</sup>	10	10	10	10 <sup>d</sup>	—	
1–3 years	10 <sup>e</sup>	10	20	15	10	15	
4–6 years	10	10	20	15	10	15	
7–8 years	10	10	20	15	10	15	
9–10 years	10	10	20	15	10	15	
11–14 years	10	10	20	15	10	15	
15–17 years	10	10	20	15	10	15	
18–64 years	10	10	20	15	10	15	
65+ years	10	10 (20 for 75+years)	20	15	10	15 (20 for 70+ years)	
Pregnancy	10	10	20	15	10	15	
Lactation	10	10	20	15	10	15	

<sup>a</sup>Those that established under conditions of minimal cutaneous vitamin D synthesis. Note: The Health Council of the Netherlands also re-evaluated their vitamin D DRV in 2012, but included sunlight contribution in the recommendations and thus we did not include.

<sup>b</sup>To convert vitamin D reference intakes from µg/day to IU/d, multiply by 40.

<sup>c</sup>Set as a Safe Intake; set for some nutrients if there were insufficient data to set DRVs. They are judged to be a level or range of intake at which there is no risk of deficiency, and below a level of where there are undesirable effects.

<sup>d</sup>Set as Adequate Intake.

25(OH)D, 25-hydroxyvitamin D; AI, Adequate Intake; DRI, Dietary Reference Intakes; DRV, Dietary Reference Values; EAR, Estimated Average Requirement; RDA, Recommended Dietary Allowance; RNI, Reference Nutrient Intake.

healthy vitamin D status all year round. Thus, the importance of vitamin D nutrition is the corollary of UVB exposure deficit [22]. This is problematic from a public health perspective, as there is considerable evidence that the dietary supply is currently unable to offset the UVB exposure deficit, which increases with latitude and the duration of winter [23], and aggravates other factors contributing to low vitamin D status, such as clothing customs. There are very few rich natural sources of vitamin D; these are oily fish and fish liver oil (which are consumed sporadically), egg yolk, and fortified foods, and vitamin D<sub>2</sub> is found in UV-exposed mushrooms or UV-irradiated yeast (see Chapter 55). Illustrated by analysis of the United Kingdom's food composition database, it is clear to see how scarce vitamin D is in the food supply [24,25]. Only 1% of food codes contain vitamin D in the range of 5–10 and >10 µg/100 g, respectively, and 3% contain 1.5–5 µg/100 g. Fish and fish products make up 70%–78% of the foods containing >5 µg/100 g and 35% of those containing 1.5–5 µg/100 g. Eggs and egg products and meat and meat products make up 17% and 10% of foods with 1.5–5 µg/100 g, respectively [24]. Given the low

consumption of fish and inaccessibility of naturally rich sources of vitamin D for many communities, as well as the global drive toward a more plant-based diet, this low supply is likely to be reduced further. Therefore, for many reasons, it is unrealistic to expect the usual diet of many countries to supply vitamin D at 10–20 µg/day across the population.

#### 1.4 Are dietary supplements the solution?

Vitamin D supplementation is often recommended to close this gap. In some countries, vitamin D supplement use has been recommended as national policy, particularly for at-risk population groups [26,27], especially infants [28,29]. This is not surprising in light of the tangible lines of evidence that vitamin D supplementation can significantly improve vitamin D intake and status [30] (see Chapter 56). For example, there have been a number of systematic reviews and metaanalyses of vitamin D randomized controlled trials (RCTs), which highlight the effectiveness of vitamin D supplementation in improving vitamin D status across a variety of age,

race, ethnic, and gender groups [31–34]. Beyond the controlled setting of RCTs, at a population level, there is evidence from nationally representative surveys that show relatively increased vitamin D status and a lower prevalence of vitamin D deficiency among participants stratified by consumption of vitamin D–containing supplements. For example, data from NHANES 2011–14 ( $\geq 1$  year,  $n = 16,180$ ) showed that the prevalence of serum 25(OH)D  $< 30$  nmol/L was 6.9% in nonsupplement users compared with 1.1% in supplement users [5]. However, relying on supplements as a public health strategy to increase intakes across the population has intrinsic limitations. Supplements are only effective in those who consume them, and uptake is usually lower than  $\sim 40\%$  overall, and highest among infants and older adults rather than children, adolescents and young adults, who are at high risk of deficiency [35–37].

The other consideration is safety. It is important to remember that vitamin D is a nutrient, and many authors now acknowledge that it is best taken in moderate amounts on a frequent, regular basis. Intermittent, high-dose regimens only correct deficiency in the short term and may have unintended adverse effects [38]. There is also potential for overdosing, particularly when supplement consumers are not under medical supervision. For example, the NHANES assessment of trends in supplementation shows that daily supplemental vitamin D intake of 100  $\mu\text{g}$  (the current tolerable upper intake level [UL] for vitamin D in adults [14]), or more, prior to 2005–06 was less than 0.1%, but thereafter climbed to 3.2% in 2013–14 [39]. While we acknowledge the usefulness of supplements under medical supervision for the rapid correction of clinical deficiency (serum 25(OH)D  $< 30$  nmol/L), risk management strategies for public health must be designed to meet the needs of the unsupervised majority, on an ongoing basis.

## 1.5 Food-based strategies are required

Thus, there is a need for sustainable food–based strategies to bridge the gap between current and recommended intakes of vitamin D to minimize the prevalence of low serum 25(OH)D concentrations, without increasing the risk of habitual excessive intakes. The distribution of vitamin D intakes is heavily skewed to the left, with a small number of high-dose supplement users at risk of excessive intakes [40]. From a public health perspective, we need to work toward changing the shape of the vitamin D intake distribution, thereby increasing intakes in the lower 50%, without changing the proportion with intakes close to the UL of 100  $\mu\text{g}/\text{day}$  [40]. Careful application of fortification (nutrient addition in controlled amounts) and biofortification (i.e., enrichment of animal food sources, such as meats, eggs, fish, vegetables,

mushrooms, by fortifying animal feed, and/or UVB irradiation of food products) could safely increase vitamin D in the usual food supply, thereby increasing intakes of vitamin D across the distribution without risking excessive dosing. Many countries have opted for mandatory or voluntary food fortification, with variable success [30,41,42]. The rest of this chapter will briefly overview the current dietary supply of vitamin D and illustrate the gap that exists between this supply and the dietary targets, and then consider the role of vitamin D–fortified and vitamin D–biofortified foods in tackling low vitamin D intakes. This will include the clinical evidence for efficacy of fortified and biofortified foods, as well as provide a brief discussion of some of the technical issues around fortification and biofortification, including the forms of vitamin D that can be used, addition amounts, and food vehicles. Finally, the chapter will highlight the types of studies/analysis/modeling required prior to initiating public health measures.

## 2. Current intakes of vitamin D in young and adult populations in North America and Europe and how these compare against dietary targets

We have previously provided a summary of vitamin D intakes and sources in adults and children, focusing on data from North America and Europe, which showed that intakes of vitamin D are inadequate ([23], and see Tables 58.2 and 58.3), and newer data have been reported in the past few years confirming these observations. In 2018, the national nutrition surveys in the 53 countries of the WHO European region were summarized, and the nutrient intake data (excluding that from supplements) were provided [43,44]. Vitamin D intakes from data collected between 2004 and 2015 in 21 countries from Northern, Western, Southern, and Central and Eastern Europe were available for adults. Intakes were low on average, at 2.7 and 3.3  $\mu\text{g}/\text{day}$  among females and males, respectively. Similarly, intake data from 13 countries across Europe collected between 2003 and 2016 were available for children; average intakes were 2.7 and 2.2  $\mu\text{g}/\text{day}$  for those aged  $< 10$  and  $\geq 10$  years, respectively. Regional differences were evident, with a general trend for higher intakes in the North compared with Central and Eastern Europe. Notwithstanding the differences introduced by varying methods of data collection and the coverage and accuracy of the food composition data, among the surveys that captured the relevant data, the main source of variation was the contribution from nutritional supplements [23]. This can increase mean intake values, providing a misleading view of the prevalence of low intakes. For example, the National Adult Nutrition Survey in Ireland can stratify nutrient intake data according to vitamin D

**TABLE 58.2** Vitamin D intakes ( $\mu\text{g}/\text{d}$ ) in adults from selected national nutrition surveys and cohort studies.

Country	Survey	Year	Sex	Age group (y)	N	Mean (SD) ( $\mu\text{g}$ )	Median (IQR) ( $\mu\text{g}$ )	Sources	National fortification policy
USA	NHANES	2003–06	Men	19–30	549	6.6 (9.4)	NR	All	Milk, mandatory; other foods voluntary
						5.1 (7.0)	NR	Food	
				31–50	758	7.9 (8.3)	NR	All	
						5.4 (8.3)	NR	Food	
				51–70	614	8.8 (9.9)	NR	All	
						5.1 (7.4)	NR	Food	
				$\geq 71$	368	10.7 (13.4)	NR	All	
						5.6 (7.7)	NR	Food	
			Women	19–30	481	5.8 (6.6)	NR	All	
						3.6 (6.6)	NR	Food	
				31–50	693	7.7 (13.2)	NR	All	
						4.4 (7.9)	NR	Food	
				51–70	610	10.1 (24.7)	NR	All	
						3.9 (9.9)	NR	Food	
				$\geq 71$	332	10.0 (9.1)	NR	All	
						4.5 (3.6)	NR	Food	
USA	NHANES	2003–06	Both	$\geq 19$	8860	8.1 (18.9)	5.8 (3.1, 12.1)	All	Milk, mandatory; other foods voluntary
			Both	$\geq 19$		4.5 (9.4)	3.9 (2.5, 5.8)	Food	
			Both	$\geq 19$		2.0 (3.8)	1.8 (1.2, 2.6)	Base diet	
Canada	CCHS-nutrition	2015	Men	19+	5667	5.1 (7.5)	4.5 (3.1, 6.5)	Food	Milk and margarine
				19–30	765	5.1 (5.5)	4.7 (3.2, 6.5)	Food	
				31–50	1837	5.1 (4.3)	4.6 (3.2, 6.5)	Food	
				51–70	1961	5.0 (4.4)	4.5 (3.1, 6.4)	Food	
				>70	1104	5.0 (6.6)	4.5 (3.1, 6.3)	Food	
			Women	19+	6317	4.2 (15.9)	3.7 (2.5, 5.4)	Food	Mandatory
				19–30	815	4.1 (8.6)	3.7 (2.5, 5.4)	Food	
				31–50	2055	4.2 (9.1)	3.8 (2.5, 5.4)		
				51–70	2107	4.3 (4.6)	3.9 (2.6, 5.5)		
				>70	1340	4.3 (3.7)	3.8 (2.6, 5.5)	Food	
UK	NDNS	2016/17–18/ 19	Men	19–64	570	5.2 (21.2)	2.7	All	Margarine
						3.2 (2.7)	2.5	Food	
				$\geq 65$	193	7.3 (11.4)	4.5	All	
						3.7 (2.4)	3.3	Food	
			Women	19–64	822	5.5 (15.4)	2.6	All	mandatory; other foods voluntary
						2.6 (1.9)	2.2	Food	
				$\geq 65$	259	8.3 (14.3)	3.8	All	
						2.8 (1.9)	2.6	Food	

Continued



**TABLE 58.2** Vitamin D intakes (µg/d) in adults from selected national nutrition surveys and cohort studies.—cont'd

Country	Survey	Year	Sex	Age group (y)	N	Mean (SD) (µg)	Median (IQR) (µg)	Sources	National fortification policy
Ireland	NANS	2008–10	Men	18–64	634	4.6 (7.1)	2.9 (NR)	All	Margarine
			Men	18–64	634	3.4 (2.8)	2.6 (NR)	Food	mandatory;
			Women	18–64	640	3.9 (5.2)	2.6 (NR)	All	other foods
			Women	18–64	640	2.8 (2.1)	2.3 (NR)	Food	voluntary
			Men	≥65	106	5.2 (4.5)	4.0 (NR)	All	Margarine
			Men	≥65	106	4.3 (3.7)	3.3 (NR)	Food	mandatory;
			Women	≥65	120	8.5 (13.6)	3.9 (NR)	All	other foods
			Women	≥65	120	3.4 (2.6)	2.9 (NR)	Food	voluntary
WHO-Europe (21 countries)	Rippin et al. [43] review	2004–15	Men	Adult	—	3.3 (NR)	NR	Food	Various
West			Women	Adult	—	2.7 (NR)	NR	Food	
			Men	Adult	—	3.5 (NR)	NR	Food	
Central and Eastern			Women	Adult	—	2.8 (NR)	NR	Food	
			Men	Adult	—	1.5 (NR)	NR	Food	
North			Women	Adult	—	1.1 (NR)	NR	Food	
			Men	Adult	—	7.8 (NR)	NR	Food	
			Women	Adult	—	6.1 (NR)	NR	Food	

CCHS, Canadian Community Health Survey; IQR, interquartile range; NANS, National Adult Nutrition Survey; NDNS, National Diet and Nutrition Survey; NHANES, National Health and Nutrition Examination Survey; NR, not reported; SD, standard deviation.  
Modified from Ref. [23].

**TABLE 58.3** Vitamin D intakes (µg/d) in children and adolescents from national nutrition surveys.

Country	Survey	Year	Sex	Age group (y)	N	Mean (SD) (µg)	Median (IQR) (µg)	Sources	National fortification policy
USA	NHANES	2003–06	Boys	4–8	431	9.3(8.3)	NR	All	Milk, mandatory;
						6.4 (6.2)	NR	Food	other foods
			Boys	9–13	522	7.5 (16.0)	NR	All	voluntary
						5.7 (4.6)	NR	Food	
			Boys	14–18	654	6.9 (12.8)	NR	All	
						6.1 (10.2)	NR	Food	
			Girls	4–8	468	7.9 (13.0)	NR	All	
						5.5 (6.5)	NR	Food	
			Girls	9–13	525	7.7 (22.9)	NR	All	
						5.3 (13.7)	NR	Food	
			Girls	14–18	643	5.0 (12.7)	NR	All	
						3.8 (5.1)	NR	Food	

**TABLE 58.3** Vitamin D intakes ( $\mu\text{g}/\text{d}$ ) in children and adolescents from national nutrition surveys.—cont'd

Country	Survey	Year	Sex	Age group (y)	N	Mean (SD) ( $\mu\text{g}$ )	Median (IQR) ( $\mu\text{g}$ )	Sources	National fortification policy
USA	NHANES	2009–12	Both	2–8	2871	9.4 (NR)	7.8 (5.4, 11.7)	All	Milk, mandatory;
						7.3 (NR)	6.8 (4.9, 9.1)	Food	other foods
						1.3 (NR)	1.2 (0.9, 1.7)	Base diet	voluntary
USA	NHANES	2009–12	Both	9–18	3238	6.9 (NR)	5.6 (3.8, 8.1)	All	Milk, mandatory;
						5.7 (NR)	5.2 (3.6, 7.2)	Food	other foods
						1.3 (NR)	1.2 (0.9, 1.7)	Base diet	Voluntary
Canada	CCHS 2.2	2004	Both	1–8	5655	6.2 (7.5)	5.6 (4.1, 7.5)	Food	Milk and
			Boys	9–18	4536	7.3 (6.7)	6.9 (4.9, 9.4)	Food	margarine
			Girls	9–18	4406	5.4 (6.6)	5.0 (3.7, 6.8)	Food	mandatory
UK	NDNS	2016/17–2018/19	Boys	4–10	372	3.7 (3.6)	2.5	All	Margarine
						2.4 (1.4)	2.1	Food	mandatory;
						3.0 (4.4)	2.1	All	other foods
			Girls	4–10	353	2.4 (1.7)	1.9	Food	voluntary
						3.6 (9.0)	2.3	All	
						2.3 (1.5)	2.0	Food	
						2.9 (3.5)	1.9	All	
Netherlands	DNFCS	2005–06	Both	2–3	640	4.4	3.8 (2.1, 6.3)	All	Voluntary
						1.8	1.8 (1.4, 2.2)	Food	
						2.7	2.3 (1.7, 3.2)	All	
						2.0	2.0 (1.5, 2.4)	Food	
Europe	ENHR	2009	Boys	4–6	2541	1.8–5.8	NR	Mixed	Various
			Girls	4–6	2295	1.5–6.5	NR	Mixed	
			Boys	7–9	2736	1.5–6.4	NR	Mixed	
			Girls	7–9	2597	1.5–5.1	NR	Mixed	
			Boys	10–14	4069	1.5–4.8	NR	Mixed	
			Girls	10–14	4056	1.2–4.5	NR	Mixed	
WHO-Europe (16 countries)	Rippin et al. [44] review	2003–16	Boys	<10	—	2.6 (NR)	NR	Food	Various
			Girls	<10	—	2.7 (NR)	NR	Food	
			Boys	$\geq 10$	—	2.4 (NR)			
			Girls	$\geq 10$	—	2.0 (NR)			
West			Boys	<10	—	3.3 (NR)	NR	Food	
			Girls	<10	—	3.5 (NR)	NR	Food	

Continued

**TABLE 58.3** Vitamin D intakes ( $\mu\text{g}/\text{d}$ ) in children and adolescents from national nutrition surveys.—cont'd

Country	Survey	Year	Sex	Age group (y)	N	Mean (SD) ( $\mu\text{g}$ )	Median (IQR) ( $\mu\text{g}$ )	Sources	National fortification policy
Central and Eastern			Boys	$\geq 10$		2.8 (NR)			
			Girls	$\geq 10$		2.3 (NR)			
			Boys	$< 10$	—	1.1 (NR)	NR	Food	
			Girls	$< 10$	—	1.0 (NR)	NR	Food	
			Boys	$\geq 10$		1.2 (NR)			
			Girls	$\geq 10$		1.0 (NR)			
North			Boys	$< 10$	—	3.3 (NR)	NR	Food	
			Girls	$< 10$	—	3.0 (NR)	NR	Food	
			Boys	$\geq 10$		3.7 (NR)			
			Girls	$\geq 10$		2.9 (NR)			
Australia	Children's survey	2007	Boys	4–8	NR	3.0	NR	Food	Margarine and
			Boys	9–13	NR	3.4	NR	Food	fat spreads
			Boys	14–16	NR	4.0	NR	Food	mandatory;
			Girls	4–8	NR	2.7	NR	Food	dairy, voluntary
			Girls	9–13	NR	2.7	NR	Food	
			Girls	14–16	NR	2.8	NR	Food	
Ireland	NCFS II	2017–18	Boys	5–12	300	4.4 (3.1)	3.7	All	Milk, mandatory;
						3.5 (2.0)	3.1	Food	other foods
			Girls	5–12	300	4.1 (3.0)	3.3	All	voluntary
						3.1 (1.8)	2.7	Food	
Ireland	NTFS II	2019–20	Boys	13–18	212	4.1 (3.2)	3.3	All	Milk, mandatory;
						3.6 (2.2)	3.1	Food	other foods
			Girls	13–18	216	3.3 (2.7)	2.6	All	voluntary
						2.6 (1.7)	2.2	Food	

CCHS, Canadian Community Health Survey; DNFCs, Dutch National Food Consumption Survey; ENHR, European Nutrition and Health Report 2009; Australian National Children's Nutrition and Physical Activity Survey; IQR, interquartile range; NCFS-II, National Children's Food Survey II; NDNS, National Diet and Nutrition Survey; NHANES, National Health and Nutrition Examination Survey; NR, not reported; NTFS-II, National Teens' Food Survey II; SD, standard deviation.

Modified from [23].

intakes from the base diet, the contribution from the fortification component, and the intake from supplements [45]. In the 2009 survey, fortification increased median [IQR] vitamin D in the base diet from 2.1 [1.8]  $\mu\text{g}$  to 3.7 [2.9]  $\mu\text{g}/\text{day}$  among fortified food consumers, who represented the majority (60%). With a 16% prevalence of vitamin D supplement use, consumers had a median [IQR] daily intake of 8.7 [7.2]  $\mu\text{g}$ , which increased the population mean to 5.0  $\mu\text{g}$  (the median was 3.5 [3.7]). Although 32% of supplement users had intakes  $> 10 \mu\text{g}/\text{day}$ , this had almost no effect on majority, with 95%  $< 10 \mu\text{g}$ . Many surveys still do not collect supplement data, and even fewer can disaggregate the fortification component, making it difficult to

disentangle the real sources of vitamin D and the accuracy of inadequacy estimates.

In North America, NHANES 2011–14 ( $\geq 1$  year,  $n = 16,180$ ) reported a mean vitamin D intake (excluding supplements) of 4.9  $\mu\text{g}/\text{day}$  [5]. The prevalence of serum 25(OH)D  $< 30 \text{ nmol}/\text{L}$  was 7.1%, 4.7%, and 2.6% in those with vitamin D intake in the range 0–2.0, 2.1–5.1, and  $> 5.1 \mu\text{g}/\text{day}$ , respectively [5]. In NHANES 2009–12, vitamin D intakes from all sources (naturally occurring in foods plus enriched/fortified plus dietary supplements) were 14.2, 9.5, and 8.3  $\mu\text{g}/\text{day}$  for non-Hispanic White, non-Hispanic Black, and Hispanic population groups, respectively [46,47]. The Canadian Health Measures Survey 2015 ( $\geq 19$  years,

$n = 11,992$ ) [48] reported mean intakes of vitamin D of 5.1 and 4.2  $\mu\text{g}/\text{day}$  for males and females, respectively, from food sources only. This is noteworthy as in 2015, approximately 34% of Canadians took a vitamin D supplement, increasing from  $\sim 25\%$  to 30% in 31 to 50-year-olds to 37%–46% in adults aged 71 years and older [49]. Data from the 2004 cycle of the survey included children, with intakes in the range of 7–9  $\mu\text{g}/\text{day}$  in 1- to 18-year-olds [50]. Intakes among adults were higher (7.9 and 6.1  $\mu\text{g}/\text{day}$  for males and females, respectively, food sources only) in the 2004 survey [50] than the 2015 estimates [48].

As outlined before, the EAR for vitamin D is designed to meet the needs of 50% of the individuals in a life stage and gender group. This enables the evaluation of nutrient intakes on a population basis, as comparing the population intakes with the EAR provides an estimate of the prevalence of inadequate intakes. The absence of an EAR value means that evaluations of nutrient intakes in populations are subjective and difficult to interpret. While the prevalence of vitamin D intakes less than the US EAR of 10  $\mu\text{g}/\text{day}$  [14] was not provided in the collection of nutrition surveys of adults and children in countries within the WHO European region, it is clear from the mean intakes, which are typically below 5  $\mu\text{g}/\text{day}$ , that every country (with the exception of Finnish adults) has high majority with inadequate vitamin D intakes. Roman Vinas et al. [51] showed that of European national nutrition surveys reporting vitamin D intake data from 2000 onward, 77%–100% and 55%–100% of adults (19–64 years) and elderly ( $>64$  years), respectively, had vitamin D intakes below the EAR. In the United States, data from the NHANES 2009–12 showed that 72.5% of individuals (aged  $\geq 2$  years) had vitamin D intakes  $<$  EAR [46], whereas data from the Canadian Health Measures Survey 2015 ( $\geq 19$  years) suggest 94% and 98%, of males and females, respectively, had intakes  $<$  EAR [48]. Despite some small differences in the estimates, the overall picture illustrates beyond a doubt that the current dietary supply of vitamin D makes it unfeasible for almost all children and adults in Europe and many in North America to meet the 10  $\mu\text{g}/\text{day}$  target on a population basis. New dietary strategies to increase vitamin D intakes across the population distribution are required.

### 3. Dietary strategies for increasing vitamin D intake: bridging the gap by food fortification

The World Health Organization and Food and Agriculture Organization (WHO-FAO) have proposed a number of strategies for reducing micronutrient malnutrition [52], including (1) increasing the diversity of foods consumed, (2) food fortification, and (3)

supplementation. It should be noted that the WHO-FAO have indicated that all three strategies have pros and cons as well as implementation hurdles [52].

In a general sense, increasing dietary diversity is the preferred way of improving the nutrition of a population because it has the potential to improve the intake of many food constituents—not just micronutrients—simultaneously, and is potentially the most sustainable option [52]. Increasing dietary diversity means increasing both the quantity and the range of micronutrient-rich foods consumed [52]; however, in the context of vitamin D, this is particularly challenging because there are so few rich food sources. With recent calls for a radical transformation of the global food systems, with emphasis on increased consumption of plant-based foods and reductions in dairy and meat for many, increasing the intake of naturally occurring vitamin D-rich foods and increasing the diversity of vitamin D-containing foods are arguably unlikely strategies to achieve increases in vitamin D intakes [53].

Apart from the safety considerations around unsupervised dosing elaborated earlier, relying on supplements to increase intakes in the population has intrinsic and important limitations. The WHO-FAO suggest that while micronutrient supplementation often provides the fastest improvement in the micronutrient status of individuals or targeted population, food fortification tends to have a much wider and more sustained impact [52]. Further, as the benefits are potentially large, food fortification can be a very cost-effective public health intervention. Success, however, is predicated on widespread uptake and consumption by a large proportion of the target population [52].

#### 3.1 Traditional food fortification with vitamin D—what is the evidence?

Several lines of evidence, from national diet and health survey data to RCT, show that food fortification represents the best opportunity to increase the vitamin D supply to the population [23,54]. The practice of fortifying commonly consumed foods with micronutrients became widespread around the time of the World Wars to prevent micronutrient deficiencies and to address nutrient losses during food processing. By the 1930s, fortification of milk and cod liver oil supplementation of infants was widespread for the prevention of nutritional rickets. However, quality control was inadequate, and excessive addition of vitamin D to milk resulted in hypercalcemia among young children in the United Kingdom, which effectively ceased the practice of vitamin D fortification [23]. Countries vary considerably in their fortification regulations and practices. Calvo, Whiting, and colleagues have provided an

excellent overview of the North American vitamin D fortification initiatives and regulatory frameworks [55–57]. Canada currently has mandatory fortification of infant formula and medical nutrition products, milk, and margarine with vitamin D as well as egg products if their composition is altered by processing, as stipulated by the Canadian Food and Drug Regulations [58–62] (see Table 58.4). There is also voluntary fortification of butter substitutes, goat milks, and condensed milks. From a public health perspective, data on vitamin D status of Canadians who are not supplement users indicate that ingestion of at least 1 serving/day of fortified milk is associated with increments in serum 25(OH) D levels of at least 6 nmol/L per serving from November through April compared with nonmilk consumers [55,63].

While fluid milk in the United States is not required to have vitamin D added unless the label declares that it is fortified, in practice almost all milk is fortified with vitamin D on a voluntary basis [55–57,64,65]. The types of milks, dairy, and other foods that can be fortified in the United States are shown in Table 58.4, and it is of note, in recent years, the FDA in the United States have approved an increase in amount of vitamin D that may be added as an optional ingredient to milk (as vitamin D<sub>3</sub>) and milk alternatives and yogurts made from edible plants (as

vitamin D<sub>2</sub>) [64,65]. Calvo et al. [66] and Yetley [67] have highlighted that up until the first decade of the new millennium at least, most milk products such as yogurt, butter, ice cream, sour cream, cream, cottage cheese, and most varieties of hard and soft cheeses are not routinely fortified with vitamin D, which contradicts the widely held misperception that dairy products are rich sources of vitamin D [66].

In Europe, fortification practices vary between countries and may be applied voluntarily by manufacturers or implemented by national legislation. In the United Kingdom and Ireland, all margarine sold for domestic use was previously subject to mandatory fortification with vitamin D until recently [40]; currently, most margarines and fats spreads are still fortified with variable quantities on a voluntary basis. Other foods, such as breakfast cereals and dried or evaporated milks, may also be fortified on a voluntary basis as per the 2006 European Union regulation 1925/2006/EC on the addition of vitamins and minerals to foods.

### 3.2 A national case study—Finland

The Finnish experience over the past 10 years is exemplary and responsive with respect to not only implementation, but also evaluation of vitamin D fortification

**TABLE 58.4** Foods fortified with vitamin D in North American and select Nordic countries (excluding infant formula and medical nutrition products).

Country	Type of fortification	Foods fortified with vitamin D	Amount of vitamin D added
Canada	Mandatory	Margarine	530 IU/100 g
		Milk	33–45 IU/100 mL
		Milk products: milk Powder, sterilized milk, flavored milk, skim milk, evaporated milk/options <sup>a</sup>	33–45 IU/100 mL
	Voluntary under marketing authorization <sup>c</sup>	Liquid/dried/frozen whole egg/yolk or egg white	Replenishment <sup>b</sup>
		Condensed milk and goat's milk/options	80 IU/100 mL; <i>Maximum</i>
		Milk, goat's milk	80 IU/100 mL
USA	Voluntary	Margarine	1040 IU/100 g
		Milk	331 IU/100 g
		Milk	84 IU/100 g; as vit D <sub>3</sub> ; <i>Maximum</i>
		Edible plant–based beverages and milk alternatives	84 IU/100 g; as vit D <sub>2</sub> <sup>d</sup>
		Yogurt (full fat, low-fat, no-fat)	89 IU/100 g
		Edible plant–based yogurt alternatives	89 IU/100 g; as vit D <sub>2</sub> <sup>d</sup>
		Soy beverage products	89 IU/100 g; as vit D <sub>2</sub> <sup>d</sup>
		Soy-based butter substitute spreads	330 IU/100 g; as vit D <sub>2</sub> <sup>d</sup>
		Cheese and cheese products, excluding cottage cheese, ricotta cheese	81 IU/30 g; as vit D <sub>3</sub>



**TABLE 58.4** Foods fortified with vitamin D in North American and select Nordic countries (excluding infant formula and medical nutrition products).—cont'd

Country	Type of fortification	Foods fortified with vitamin D	Amount of vitamin D added
Finland	Voluntary (but recommendation-based)	Soy-based cheese substitutes and products	270 IU/100 g; as vit D <sub>2</sub> <sup>d</sup>
		Calcium-fortified 100% fruit juices and fruit juices drinks	100 IU/240 mL; as vit D <sub>3</sub>
		Cereal products:	
		enriched farina, ready-to-eat breakfast cereal;	350 IU/100 g
		enriched rice, enriched noodle products, enriched macaroni products	90 IU/100 g
Sweden	Mandatory	Margarine and fat spreads (not butter)	800 IU/100 g
		Fluid milk products: milk, yoghurt, sour milk	40 IU/100 g
		Margarine and fat spreads	780–840 IU/100 g
		Milk: <3% fat, also lactose-free and vegetable-based alternatives	38–44 IU/100 g
Norway	Voluntary (but recommendation-based)	Sour milk: <3% fat, also lactose-free and vegetable-based alternatives	30–44 IU/100 g
		Butter and margarine	400 IU/100 g
		Low-fat milk, also lactose-free	16 IU/100 g

<sup>a</sup>Options include skim milk with added solids, partly skimmed milk with added milk solids, partly skimmed milk, skim milk powder, evaporated skim milk, concentrated skim milk, evaporated partly skim milk, and concentrated partly skimmed milk.

<sup>b</sup>Mandatory if there is a reduction in the vitamin D and/or mineral content to restore the vitamin or mineral nutrient to the amount that was present in the egg product before processing.

<sup>c</sup>Marketing Authorization (MA) for Vitamin D in Milk, Goat's Milk and Margarine: SOR/2021-278 (Canada Gazette, Part II, Volume 156, Number 2 SOR/2021-278 December 29, 2021).

<sup>d</sup>Vitamin D<sub>2</sub> mushroom powder can also be used to achieve these content levels.

Modified from [40,41,55].

policies. In 2003, the Finnish government initiated regulations for the optional vitamin D<sub>3</sub> fortification of milks and yogurt (0.5 µg/100 g) and margarine and spreads (10 µg/100 g) [68]. The resulting increases in vitamin D intakes following implementation of the fortification policy and in serum 25(OH)D concentration pre- and 1-year postimplementation in subsets of the Finnish population, particularly children and teenagers, were assessed and reported [69,70]. As one example, after fortification, the prevalence of winter vitamin D deficiency (serum 25(OH)D < 25 nmol/L) in Finnish men in the Defense Forces (18–28 years) decreased from 19% in January 2003 to 5% in January 2004 [68]. However, Lehtonen-Veromaa et al. [71] found only a modest increase in mean vitamin D intakes (from 4 to 5.4 µg/day) adolescent girls as a result of the fortification policy, and no change in serum 25(OH)D, probably due to low dairy consumption among adolescent girls. In 2010, the National Board of Nutrition doubled the 2003 recommended levels for vitamin D fortification of fluid milk products and spreads [72] (see Table 58.4). Data from the national nutrition survey in Finland (2012) reported vitamin D intakes at 11.1 µg/day among men and 12.8 µg/day among women, with milk and fat spreads contributing up to 60%, depending on age and sex

[73]. A comparison of standardized serum 25(OH)D data from the *Finnish Health* surveys in 2000 (*n* 6134) and 2011 (*n* 4051) shows that the mean serum 25(OH)D increased from 48 nmol/L to 65 nmol/L, and the prevalence of serum 25(OH)D < 30 nmol/L decreased from 12% to <1% [74].

The vitamin D fortification policies in other Nordic countries are summarized in Table 58.4 and were reviewed recently by Itkonen et al. [41]. In brief, in Sweden, milks with a fat content less than 3%, sour milk products, lactose-free products, and vegetable-based alternatives as well as margarines are mandatorily fortified with vitamin D<sub>3</sub>; in Norway, butter, margarine, and some types of low-fat milk are voluntarily fortified with vitamin D, but with lower amounts than Finland and Sweden, whereas in Denmark and Iceland, only a few products are fortified with vitamin D.

### 3.3 Hierarchy of evidence—randomized controlled trials and metaanalyses

Many RCTs of vitamin D—fortified foods have been carried out all over the world in different population groups, using diverse foods and various levels of addition of vitamin D, and a number of systematic reviews

(SR) and metaanalyses of RCTs have confirmed that food fortification is effective in improving vitamin D status [75–79]. The first SR of these trials was carried out by O'Donnell et al. [75], and we updated this analysis in 2012 [76] following publication of several studies around the world. Of 16 separate RCTs in the 2012 SR, all but two showed a significant effect of supplementation on circulating 25(OH)D, which increased by 19.4 nmol/L (95% CI 13.9, 24.9), corresponding to a 1.2 nmol/L (95% CI: 0.72, 1.68) increase for each 1 µg ingested [76]. Twelve RCTs used dairy products as a food source (nine used milk or milk powder). While this is evidence at the highest level that food fortification increases 25(OH)D, both SRs identified overreliance on milk as a weakness in the evidence and recommended considering a more inclusive approach to commodity-based fortification, with careful consideration of the foods used and levels of additions applied [75,76]. In 2018, an SR and metaanalysis of the effect of vitamin D interventions on vitamin D status in children aged 2–18 years included RCTs with fortified foods ( $n = 9$ ; the majority of which were with dairy-based foods), supplements, and bolus injections [77]. The response per 100 IU vitamin D/d was greater with fortified foods than supplements, in trials with a mean baseline serum 25(OH)D < 30 nmol/L and with baseline vitamin D intakes < 100 IU/d. In these analyses, the serum 25(OH)D response to vitamin D intake differed on the basis of baseline status, intakes, and delivery mode, but not age, sex, or latitude.

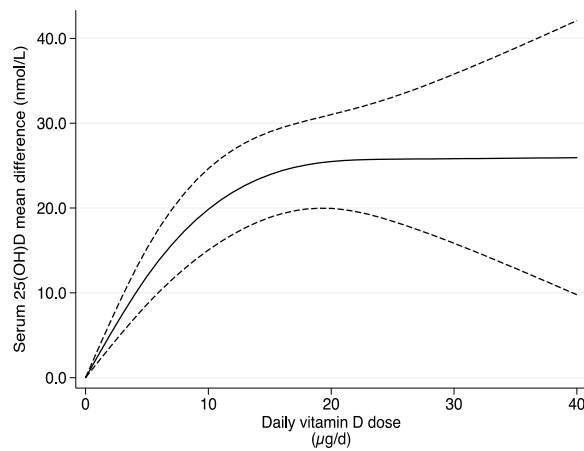
In 2021, Dunlop and colleagues published a further update and expanded the scope of earlier SRs to include adults and children, to evaluate effects by vitamin D vitamer, and to investigate linear and nonlinear dose–response relationships [78]. This review included 34 studies, which used a broad range of foods in addition to milks, including yogurt, eggs, cheese, fruit juice, biscuits, snack bars, crisp bread, and lavash bread, as well as combinations of foods such as yogurt plus cheese, or egg, yogurt, cheese plus crisp bread. Doses of vitamin D ranged from 3.3 to 100 µg/day (overall mean dose weighted by number of participants was 16.2 µg/day: adults 18.9 µg/day; children 11.8 µg/day). Data from 3930 participants including 2398 adults and 1532 children showed an overall dose–response rate of 1.71 (95% CI 1.24, 2.17) nmol/L per 1 µg of added vitamin D; rates varied by vitamer and adult/child. Thus, with a mean increase of 25(OH)D concentrations of 21.2 nmol/L (95% CI 16.2, 26.2) among participants who received fortified foods, this analysis showed consistent effects of fortifying foods in trials of moderate to high quality. Fig. 58.1 shows nonlinear dose–response effects for all studies combined, adults and children. Using restricted cubic splines, thresholds occurred at 26–29 nmol/L for doses ~20–22 µg/day. When studies

were separated by adults and children, the rate began to slow at a higher dose in adults than children.

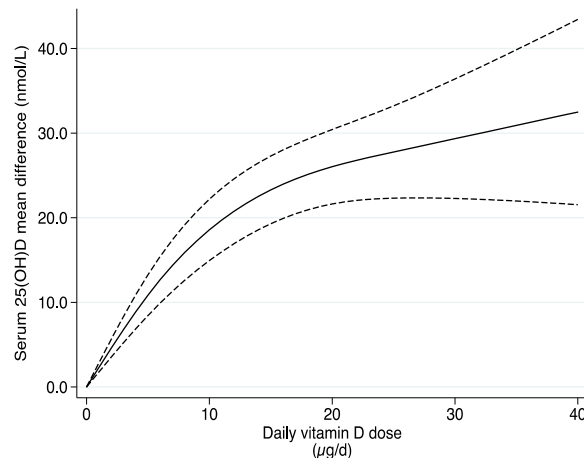
The most recent SR identified 11 RCTs with vitamin D–fortified foods with data available for an individual participant data analysis of the response of winter serum 25(OH)D to total vitamin D intake among children and adults and derived dietary requirements for vitamin D intake at prespecified 25(OH)D targets [79]. Of the 11 RCTs, 6 studies used dairy-based foods (of which 5 used a single source (cheese or a cow's milk-based beverage) and 1 used yogurt and cheese), 1 RCT each used bread; eggs; orange juice or biscuits; or milk plus bread; or a combination of four foods (vitamin D–fortified low-fat cheese, yogurt, eggs, and crisp bread). The analysis provided further evidence that food-based approaches to achieve an intake of 12 µg/day could prevent very low vitamin D status (serum 25(OH)D < 30 nmol/L) in the general population [79].

The consistencies between these different SRs are striking: it must now be considered proven that food fortification with vitamin D increases serum 25(OH)D among consumers; that increases in 25(OH)D are nutritionally significant but moderate (i.e., not at risk of excess), and consistent with regular food portions consumed. Fortification of a range of diverse products to maximize the benefit in terms of both consumer coverage and meaningful increases in 25(OH)D for public health is recommended with careful monitoring of doses and quantities consumed through the national dietary surveys supported by current food composition data. The importance of considering dietary diversity has been underscored by evidence from dietary surveys—while vitamin D–fortified milk and related dairy products (including low-fat cheese) are highly effective in raising serum 25(OH)D levels when consumed [75,76,80], and fortification of dairy products beyond fluid milk is technologically feasible [81], evidence that dairy fortification is protective against deficiency on a population basis is present only among high consumers of milk, such as young children. El-Hayek et al. [82] showed that 25(OH)D concentrations in preschool children in Montreal increased in a step-wise manner by tertile of milk intake. Among the wider population, with increased variability in milk consumption, the situation becomes more complex. In the United States, Newman et al. [46] presented the cumulative proportions of vitamin D intakes from naturally occurring food sources only, plus enrichment/fortification, plus dietary supplements in individuals aged >2 years in the NHANES 2009–12. The mean intake of 1.6 µg/day from natural food sources increased to 5.4 µg/day when enrichment/fortification was accounted for. Increases were highest among young children (1.3–7.3 µg/day in 2–8 year olds) due to their high milk intakes, dropping to an increment of 1.3–5.7 µg/

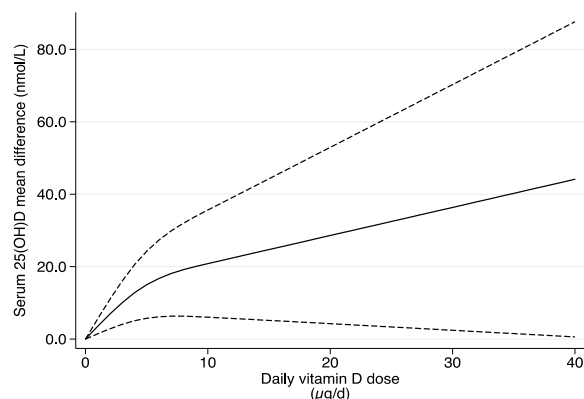
A.



B.



C.



**FIGURE 58.1** Dose-response data for the change in circulating 25(OH)D concentrations in randomized controlled trials of vitamin D

fortified and biofortified foods. Restricted cubic spline models with three knots at vitamin D doses of 0, 5 and 20  $\mu\text{g}/\text{day}$  for: (A) Overall (34 publications, 40 data points); (B) adults (27 publications, 31 data points) and (C) children (7 publications, nine data points) are shown. Modified from Ref. [78], with author's permission.

day in 9- to 18-year-olds [46]. Likewise, in Canada, mean vitamin D intakes arising from milk products in those aged 9 years and over were in the range 2.5–3  $\mu\text{g}/\text{day}$ , contributing 49.1% to the mean daily intake of vitamin D [57]. However, the prevalence of inadequate vitamin D intakes remains very high in both the United States and Canada [46,48,55–57], highlighting variable rates of milk consumption.

Even with mandatory fortification, >80% have vitamin D intakes <10  $\mu\text{g}/\text{day}$  in Canada (>94% when vitamin D intakes from food and beverages alone were considered [48]); however, modeling of the 2004 Canadian Community Health Survey 2.2 data ( $n = 34,381$ ) showed that this prevalence could be decreased to <50% in all groups with increased vitamin D levels in milk and the addition of vitamin D to cheese and yogurt at various levels [50]. In countries where fortification of milk is voluntary and the uptake is far less than in the United States, the impact of vitamin D-fortified milk and dairy on adequacy of vitamin D intake is low. Using data from recent national nutrition surveys in the United Kingdom and Ireland as two exemplars, it is evident that in general the percentage contribution that milk and dairy makes to the mean daily intake of vitamin D are low ( $\leq 7\%$ ) for those aged 11 years upward in both populations, while fat spreads contribute about 16% and 8% in the United Kingdom and Ireland, respectively [37,83]. About 17% and 38% of Irish adults, aged 18–64 years, used vitamin D-fortified milks and fat spreads, respectively, in the most recent national dietary survey of 2010 [37]. Interestingly, survey data collected 10 years previously in Ireland, when fortified milk was a rare product, showed that only 5% of adults were consumers—no doubt this figure will continue to increase over time [37]. Currently, with market penetrance and current ad hoc/nonstrategic levels of addition, it is not surprising to find that in Europe in general the intake of vitamin D from voluntary fortified foods has been reported as low [84].

The problem of fortifying a single staple, e.g., milk, or focusing on a commodity sector such as dairy, is that it does not increase the vitamin D supply in non- or low consumers [40]. Blacks in the United States have lower intakes of vitamin D than whites (9.5 vs. 14.2  $\mu\text{g}/\text{day}$ , respectively, [47]) due to their consumption of less milk, ready-to-eat cereals, and dietary supplements [56]. In addition, nondairy plant-based beverages, also known as plant milks, some of which are not fortified

with vitamin D, are an increasingly popular product category and may displace some vitamin D–fortified dairy milk within the food chain for some populations.

Thus, while acknowledging the valuable contribution fortified milk makes to vitamin D intakes among consumers, particularly in children, and the continued need for fortification of milk and other dairy products, additional strategic approaches to fortification, including biofortification, of a wider range of foods, have the potential to increase vitamin D intakes in the population [23,40]. Beyond dairy-related foods and plant-based alternatives, fruit juices and certain cereal products (but not wheat) can be fortified with vitamin D in the United States (but not by law in Canada) [55] (see Table 58.4). Not surprisingly, ready-to-eat cereals (about 75% are fortified [67]) together with milk are the predominant food sources of vitamin D in the United States [55,56].

#### 4. Consideration of other vitamin D–fortified foods

The WHO-FAO have suggested that in many settings, food fortification can be a very affordable way of correcting inadequate micronutrient intakes, and more often than not, the main challenge is finding a suitable industrially manufactured food vehicle that is consumed in sufficient amounts by the population at risk [52]. The cost and choice of food vehicle(s) are also important more considerations in terms of trying to ensure equality of access to these foods within the population. In the context of adapting the global food systems to minimize environmental impacts, with emphasis on increased consumption of plant-based foods and reductions in dairy and ruminant meat [85], the fortification of non–animal-derived foods, such as wheat and other cereals, bread, edible oils, and possibly biofortification of fungi and baker's yeast, with vitamin D may be of increasing importance for nutrition security.

In terms of diversification of food fortification beyond milk, experimental evidence from Denmark [86] in the form of data from a large, well-characterized RCT showed effects of vitamin D–fortified milk and bread on serum 25(OH)D in 201 families ( $n = 782$  children and adults, aged 4–60 years). The groups randomized to vitamin D–unfortified and vitamin D–fortified foods had median intakes of vitamin D of 2.2 and 9.6  $\mu\text{g}/\text{day}$ , respectively, over the 6 months of the study. By the end of the study period, none and 16% in the fortified food group had serum 25(OH)D levels below 25 and 50 nmol/L, respectively, with the corresponding prevalence estimates for the group receiving unfortified foods at 12% and 65%

[86]. With no evidence of an adverse effect, this is compelling evidence for efficacy of a two-strand/multiapproach to low-dose fortification of commodity foods.

To complement priority evidence of effectiveness of food fortification approaches from RCTs that evaluate their impact on reducing the prevalence of vitamin D deficiency in the populations studied [4], dietary modeling analysis, based on data from nationally representative dietary surveys, can provide *in silico* projections of how these food interventions may impact on the degree of vitamin D intake inadequacy in the population [37]. For example, Allen et al. [87] modeled the impact of a number of simulated vitamin D fortification scenarios, with milk and wheat flour identified as primary fortification vehicles, on vitamin D intake distribution within the first 2 years (2008–10) of the UK National Diet and Nutrition Survey rolling program ( $n = 2127$  individuals). At a simulated fortification of 10  $\mu\text{g}$  vitamin D/100 g wheat flour, the proportion of at-risk groups estimated to have vitamin D intakes below the UK Reference Nutrient Intake were reduced from 93% to 50% [87]. Interestingly, the simulation of the fortification of wheat flour at this concentration was more effective than that of the fortification of milk (at concentrations between 0.25 and 7  $\mu\text{g}$  vitamin D/100 L milk) or fortification of milk and flour combined. The authors suggested that vitamin D fortification of wheat flour could be a viable option for safely improving vitamin D intakes and the status of the UK population groups at risk of deficiency [87]. Finally, a similar modeling exercise was undertaken to determine the best foods for potential vitamin D food fortification for adults  $\geq 50$  years of age in Ireland [88]. Bread and milk, as the most frequently consumed foods across all meals, were targeted for the data modeling exercise. The modeling showed that fortifying milk (generally all with 1.5  $\mu\text{g}/100$  mL, but some milks currently fortified with 2.5  $\mu\text{g}/100$  mL were retained in the model) or bread (all with 5  $\mu\text{g}/100$  g) resulted in 31% and 56% of individuals, respectively, meeting a target intake of 10  $\mu\text{g}/\text{day}$ ; however, fortifying both simultaneously resulted in 71% meeting this target intake.

More globally, Babu and Calvo [89] suggested that fortification of wheat flour may have potential to alleviate vitamin D deficiency in countries where pasteurized milk is not widely consumed. Currently, four countries (Jordan, Mongolia, Palestine, and Chile) have mandatory fortification of wheat flour with vitamin D, whereas four more have voluntary fortification [90]. Nine countries mandate the fortification of edible oils with vitamin D, while an additional four countries allow voluntary fortification of oil [90]. The majority of these countries are either in Asia or Africa.



#### 4.1 Additional considerations in relation to forms of vitamin D and addition amounts for traditional fortification

As discussed before, while recent data suggest that the levels of vitamin D added to food would need to be high enough so as to ensure dietary requirements are met and health outcomes optimized, knowledge of which are the most effective forms of vitamin D to use in some of these preventative approaches is also important. The following section will briefly consider the main forms of vitamin D that could be used in terms of fortification of food.

#### 4.2 Vitamin D<sub>2</sub> versus vitamin D<sub>3</sub>

While vitamin D<sub>2</sub> and D<sub>3</sub> [the two main food-derived forms (although vitamin D<sub>2</sub> only occurs in only a minority of foods and generally at very low levels naturally) and those used in vitamin D supplements] could be used as a fortificant, there is still ongoing debate in relation to the relative efficacy of these two forms of vitamin D in terms of raising serum total 25(OH)D (i.e., concentration of serum 25(OH)D<sub>2</sub> plus 25(OH)D<sub>3</sub>) [33,91–93]. Some, but not all, studies suggest that the response of serum total 25(OH)D to increased intake of supplemental vitamin D<sub>2</sub> is lower than that of supplemental vitamin D<sub>3</sub> (reviewed elsewhere [13,14,32,33,91–93]). Houghton and Vieth [93], in their review of the available data and evidence, provide a very succinct overview of several biologically plausible mechanisms that could contribute to the greater capacity of vitamin D<sub>3</sub> over vitamin D<sub>2</sub> to maintain higher 25(OH)D concentrations over time. They suggest when the data and findings are taken together, the most plausible explanations for the greater bioefficacy of vitamin D<sub>3</sub> are conceivably due to the higher affinities of vitamin D<sub>3</sub> and its metabolites than vitamin D<sub>2</sub> for hepatic 25-hydroxylase, vitamin D–binding protein, and the vitamin D receptor and because vitamin D<sub>3</sub> is not directly metabolized to 24-hydroxyvitamin D as is vitamin D<sub>2</sub> [93]. A lower response of serum total 25(OH)D with supplemental vitamin D<sub>2</sub> may also relate to enhanced catabolism of 25(OH)D<sub>3</sub>, despite an increase in serum 25(OH)D<sub>2</sub> ([94], and see below under UVB-enhanced mushrooms in Section 5). However, it is important to stress that both forms of vitamin D unquestionably elevate serum total 25(OH)D as evidenced in several systematic reviews [32,33,92] and the fact that vitamin D<sub>2</sub> has been used as a pharmaceutical preparation in the United States for decades [93]. Thus, both forms could be used as a food fortificant, especially in countries where non-animal-derived sources may be preferred. As mentioned before, the FDA has approved use of vitamin D<sub>2</sub> in milk alternatives and yogurts made from edible plants [65].

#### 4.3 25-Hydroxyvitamin D metabolites

While the major metabolite of vitamin D with biological activity is 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] [14], and there is presence of minor amounts of this metabolite in some foods of animal origin (as is the case with 25(OH)D [91]; and see below); 1,25(OH)<sub>2</sub>D, and its analogs, are only used for pharmacological purposes and not typically used in healthy people to improve vitamin D status.

While 25(OH)D has also been used for pharmacological purposes, it is present in certain foods of animal origin, such as meat, offal, eggs, and to a lesser extent fish (for reviews, see Refs. [91,95]). However, some of this contribution stems from the fact that the UK (and others such as Danish and Swiss) food composition tables suggest that 25(OH)D may possess up to five times the activity of native vitamin D<sub>3</sub> in food [25,96,97]. The “total vitamin D activity” of foods in such food compositional data accounts for not only the vitamin D content of the food but also the content of the 25(OH)D multiplied by the factor of 5. The US food composition database has recently reported 25(OH)D<sub>3</sub> data for beef and a limited number of other foods [98], but does not include these in the total vitamin D values for these foods. Taylor et al. [99] performed modeling to include overall food-derived 25-hydroxyvitamin D in intake estimates for US adults, which showed that there was a potentially meaningful increase (1.7–2.9 µg or 15%–30% of the EAR) in vitamin D intake estimates. However, there is some debate around use of this factor of 5, with alternate suggested factors ranging from 1 to 9 (for reviews, see Refs. [91,95,100]). A relatively recent review of nine available RCTs comparing physiologic doses of oral vitamin D<sub>3</sub> with oral 25(OH)D<sub>3</sub> concluded that 25(OH)D<sub>3</sub> was 3.2-fold more potent than oral vitamin D<sub>3</sub> [101]. Use of synthetic 25-hydroxyvitamin D (calcidiol monohydrate) as a food supplement (supplying a maximum daily intake is 10 µg/day) has very recently been approved by EFSA for individuals ≥11 years old, including pregnant and lactating women [102]. Use of 25(OH)D in animal feeds for certain species is permitted in Europe and the United States [103,104], and this can increase the content of 25-hydroxyvitamin D<sub>3</sub> in the human food chain (see biofortified foods in Section 5).

Whether vitamin D<sub>3</sub> or D<sub>2</sub>, and/or possibly 25-hydroxyvitamin D<sub>3</sub> in the future, is the fortificant of choice depending on food type and indeed consumer group, the levels of addition need to be cognizant of the tolerable upper intake levels (ULs) for vitamin D ([14,105] and see Section 6). As highlighted by the WHO-FAO, cost of the fortificant ingredient is also a consideration [52]. For food business, the cost of fortification is influenced by (1) the type of fortificant; (2) the point at which it is added to the product; (3) the need



for quality control; (4) the volumes being purchased; (5) the product category, and (6) the profit margin between and within different food categories [106]. For the breakfast cereal industry, vitamin D is one of the most expensive fortificants currently added because of the cost of the technologies required to add the vitamin, rather than the cost of the vitamin per se. However, the cost will be dependent on the nature of the technology needed and whether it is D<sub>2</sub> or D<sub>3</sub> that is added. For example, the cost of adding vitamin D is less for dairy-based foods and so is not perceived to be a major economic hurdle [106]. While overall, adding extra vitamin D into foods will incur a cost, for the most part this is likely to be low, at least for the consumer. For example, flour fortification with vitamin D at 550 IU [13.7 µg]/kg of flour only adds a very modest cost of US\$0.04–0.05 per metric ton of flour (Personal communication Quentin Johnson, Food Fortification Initiative, [www.ffinetwork.org](http://www.ffinetwork.org)). In the case of the 25(OH)D biofortification of eggs, the cost per dozen eggs was deemed minimal (approximately 0.1 p per dozen eggs) [106].

## 5. Vitamin D—biofortified foods

While traditional fortification practices in which exogenous vitamin D is added to dairy and other foodstuffs will continue to be an important approach for increasing vitamin D in the food supply, the use of vitamin D “biofortified” (also referred to as “bioaddition” [55]) foods has received increasing attention over the past decade [40,54]. In this approach, the animal produce (such as, for example, cultured/farmed fish, beef, pork, lamb, chicken, and eggs) could have increased vitamin D and/or 25-hydroxyvitamin D contents by virtue of addition of vitamin D and/or 25-hydroxyvitamin D (where permissible) to the respective fish, livestock, or poultry feeds. In biofortification, the vitamin D compounds in the resulting foodstuffs from the animals will be incorporated in a manner similar to native vitamin D and, unlike in traditional fortification, will be under some degree of biological regulatory control mechanisms in the animal. An important concept in relation to vitamin D biofortification is that it can increase the “total vitamin D activity” (as discussed before) of the biofortified foods. Improving the total vitamin D activity of these foods may be of consequence not only to the population as a whole, but in particular, for low or nonconsumers of vitamin D—fortified dairy products and also ethnic subgroups. For example, in the Irish adult diet, the meat, fish, egg food groups, even without biofortification, collectively contribute ~40% to the mean daily intake of vitamin D [45]. Van Horn et al. [107] reported that in US adolescent girls, while the meat and bean food group contributed ~4% to the mean daily intake of vitamin D

in white girls, it contributed 26% to the mean daily intake of vitamin D in African American girls. The possibility of enhancing the vitamin D activity of meat, eggs, and other relevant foods further by biofortification and its potential impact on population and ethnic subgroup intake estimates is of significant public health nutrition relevance.

The most researched vitamin D—biofortified food to date is, without doubt, eggs. In the past two decades, there have been several reports illustrating that the vitamin D<sub>3</sub> content of eggs can be significantly increased by the greater addition of vitamin D<sub>3</sub> to the feed of hens [108–113]; however, several of these studies [109,110,112,113] used levels of inclusion above the upper allowable level for feeds in Europe (3000 IU/kg diet [114]). Addition of commercially available 25-hydroxyvitamin D<sub>3</sub> to the diet of hens has also been shown to increase egg 25-hydroxyvitamin D<sub>3</sub> content [111–113], albeit two studies [111,112] used 25-hydroxyvitamin D<sub>3</sub> at levels above the upper allowable level for the United States and Europe (0.069 and 0.080 mg/kg diet, respectively [103,104]). Many of these studies were predominantly focused on the effect that higher levels of vitamin D compounds in animal feeds may have on the welfare of the animal itself or the quality of their produce (e.g., egg shell strength, laying rate) rather than its impact on the vitamin D activity of the resulting eggs and their potential to improve the vitamin D status of humans. The WHO-FAO have suggested that access to and use of fortificants that are well absorbed yet do not affect the sensory properties of foods is important [52]. In terms of producing eggs that would be acceptable for human consumption, addition of vitamin D and/or 25-hydroxyvitamin D<sub>3</sub> to feedstuffs at levels adhering to the maximum allowable EU regulation [103,114] has been shown to result in eggs with increased vitamin D activity (providing ~4–5 µg/egg), and, importantly, no deterioration of consumer acceptability of the biofortified eggs compared with usual eggs [106,115]. Thus, such vitamin D—biofortified eggs could supply close to half the EAR for vitamin D. As mentioned before, evidence of effectiveness of food fortification approaches from RCTs, which evaluate their impact on reducing the prevalence of vitamin D deficiency in the populations studied, is a key priority in terms of the evidence base [4]. In that context, we have shown in a winter-based RCT of older adults (*n* = 55) that weekly consumption of seven vitamin D—biofortified eggs, produced by hens provided with feed containing either vitamin D<sub>3</sub> or 25-hydroxyvitamin D<sub>3</sub> at the allowable maximum content, prevented the typical decline in serum 25(OH)D concentration during winter and any incidence of vitamin D deficiency [115]. The control group in the study, who were requested to consume weekly up to a maximum of two commercially available eggs, had a significant

decline in serum 25(OH)D over the 8 weeks of winter, and 22% had serum 25(OH)D < 25 nmol/L at endpoint [115]. From a dietary guideline perspective, the general population can include up to seven eggs a week in their diet [116], and our RCT showed no difference in serum total cholesterol among control and vitamin D—biofortified egg groups [115]. Recently, another RCT of egg consumption on vitamin D status added further support to their utility as a means of maintaining serum 25(OH)D during winter. In a 12-week, Australian-based RCT, adults (aged 25–40 years) were randomized to consume 2, 7, or 12 commercially available eggs per week [117]. This translated into weekly vitamin D doses of 10.4, 36.3, and 62.2 µg, respectively. Mean serum 25(OH)D did not change significantly in either the 7 or 12 eggs/week group, but decreased by 28.6 nmol/L in the control group after the 12 weeks of autumn/winter [117]. Serum lipids did not differ between the groups. The authors concluded that consuming seven eggs per week for 12 weeks was effective for attenuating the wintertime decline in vitamin D status in young Australian adults, with 12 eggs per week not providing any additional benefits [117].

The feasibility of producing other vitamin D—biofortified animal-based foods (such as beef, pork, and cultured/farmed fish) has been demonstrated, with variable levels of improvement in vitamin D content over nonbiofortified equivalent foods (see

Table 58.5). In relation to biofortified meats, the increments in vitamin D content are relatively modest [118–121], and this is likely due to the fact that the transfer of additional vitamin D in feedstuffs to tissues is subject to biological regulatory control mechanisms in the animal. Data from RCTs investigating the impact of consumption of vitamin D—biofortified meats on vitamin D status are as yet lacking. The UK food composition database suggests that the flesh (fillet) of farmed and wild salmon has 4.7 and 8.6 µg/100 g, respectively [25]. Graff et al. recently compared the impact of consumption of biofortified versus control salmon (fish feed had 2.6–2.9 mg vitamin D<sub>3</sub>/kg vs. 0.23 mg vitamin D<sub>3</sub>/kg, respectively) on vitamin D status in a 12-week intervention trial in healthy postmenopausal women [122]. The women consumed 150 g of salmon two times per week, with the vitamin D—biofortified and control groups consuming the vitamin D daily equivalent of 16 µg/day versus 3.9 µg/day, respectively, from the salmon. There was a significant increase in mean serum 25(OH)D of ~12 nmol/L in the vitamin D—biofortified groups versus a nonsignificant 1.3 nmol/L decline in the low vitamin D group over the 12-week intervention [122]. It should be noted, however, that the level of addition of vitamin D<sub>3</sub> to the fish feed exceeded that allowable currently by European legislation (1.5 mg/kg diet [123]). Following the demonstration of efficacy of some of these vitamin D—biofortified foods, at least, on

**TABLE 58.5** Overview of foods with increased vitamin D content through bio-fortification, either through animal feed fortification or UVB irradiation.

Vitamin D-biofortified foods [Reference(s)] <sup>a</sup> [means of enhancement]	Vitamin D content achievable (µg/100 g <sup>b</sup> )	Evidence of effectiveness	Other information
Eggs [106,115,117]:			
• 1500 IU vit D <sub>3</sub> /kg feed	2.8/egg	Winter-based RCT of older adults: Weekly consumption of 7 vitamin D-biofortified eggs prevented the typical decline in serum 25(OH)D concentration during winter and any incidence of vitamin D deficiency.	Consumer acceptability trial showed sensory parameters similar across treatments
• 3000 IU vit D <sub>3</sub> /kg feed	3.8/egg		
• 1500 vit D <sub>3</sub> + 1500 25OHD <sub>3</sub> /kg feed	4.8/egg		
• 3000 5OHD <sub>3</sub> /kg feed	5.1/egg		
In commercial setting:			
• 3000 IU (75 µg) vit D <sub>3</sub> /kg feed	Eggs from 3000 IU 25OHD <sub>3</sub> /kg feed had 43% higher total vitamin D content compared to baseline; no change from baseline in other two dietary groups (~4 µg/egg)	RCT planned for next phase of project	Sensory tests planned for next phase of project
• 1500 (37.5 µg) vit D <sub>3</sub> + 1500 (37.5 µg) 25OHD <sub>3</sub> /kg feed			
• 3000 IU (75 µg) 25OHD <sub>3</sub> /kg feed			
Beef [119–121]:			
Heifers:			
• 0 IU vit D <sub>3</sub> /kg feed	0.55		

*Continued*

**TABLE 58.5** Overview of foods with increased vitamin D content through bio-fortification, either through animal feed fortification or UVB irradiation.—cont'd

<b>Vitamin D-biofortified foods</b>			
<b>[Reference(s)]<sup>a</sup></b> <b>[means of enhancement]</b>	<b>Vitamin D content achievable</b> <b>(µg/100 g<sup>b</sup>)</b>	<b>Evidence of effectiveness</b>	<b>Other information</b>
<ul style="list-style-type: none"> <li>• 4000 IU vit D<sub>3</sub>/kg feed</li> <li>• 4000 IU vit D<sub>2</sub>/kg feed</li> <li>• 4000 IU vit D<sub>2</sub><sup>a</sup>/kg feed</li> </ul> <p>(<sup>a</sup>from UV mushrooms)</p> <p>Bulls:</p> <ul style="list-style-type: none"> <li>• 1000 IU vit D<sub>3</sub>/kg feed</li> <li>• 4000 IU vit D<sub>3</sub>/kg feed</li> </ul> <p>Pork [118]:</p> <ul style="list-style-type: none"> <li>• 50 mg vit D<sub>3</sub>/kg feed</li> <li>• 50 mg 25OHD<sub>3</sub>/kg feed</li> <li>• 50 mg vit D<sub>2</sub>/kg feed</li> <li>• 50 mg vit D<sub>2</sub><sup>a</sup>/kg feed</li> </ul> <p>(<sup>a</sup>from UV mushrooms)</p> <p>Atlantic Salmon [122]:</p> <ul style="list-style-type: none"> <li>• 2.9 mg vit D<sub>3</sub> (+4.3 mg K<sub>1</sub>)/kg feed</li> <li>• 2.6 mg vit D<sub>3</sub> (+&lt;0.001 mg K<sub>1</sub>)/kg feed</li> <li>• 0.23 mg vit D<sub>3</sub> (+4.7 mg K<sub>1</sub>)/kg feed</li> </ul> <p>UV-irradiated bakers' yeast [126]:</p> <p>UV-irradiated bread [127]:</p> <ul style="list-style-type: none"> <li>• control breads (no UV)</li> <li>• white bread</li> <li>• wholemeal bread</li> <li>• flat bread</li> </ul> <p>UV-irradiated mushrooms [125,128]:</p>	<p>1.38</p> <p>1.10</p> <p>0.88</p> <p>0.5</p> <p>1.5</p> <p>0.79</p> <p>1.33</p> <p>0.51</p> <p>0.48</p> <p>0.35–0.38 mg/kg</p> <p>0.35–0.38 mg/kg</p> <p>~0.1 mg/kg</p> <p>UV-treated baker's yeast led to bread with 30.2 mg/100 g while bread made with regular baker's yeast had &lt;0.3 mg/100 g</p> <p>&lt;0.5</p> <p>2.5–2.7</p> <p>0.9</p> <p>5.3</p> <p>Variable dependent on UV-B dose applied: 0–2 J/cm<sup>2</sup> led to 0–100 mg/g dry weight Recent overview of vitamin D<sub>2</sub> yields in different mushrooms after UV irradiation recently</p>	<p>Not tested in human intervention trial</p> <p>Not tested in human intervention trial</p> <p>12-week intervention trial in healthy postmenopausal women: Consumption of 150 g of salmon two times per week; there was a significant increase in mean serum 25(OH)D of ~12 nmol/L in the vitamin D biofortified groups versus a non-significant 1.3 nmol/L decline in the low vitamin D group over the 12-week.</p> <p>8-week, winter-based RCT in healthy adult women in Finland: Consumption of bread made with UV-exposed bakers' yeast-derived vitamin D<sub>2</sub> did not improve serum 25(OH)D (poor bioavailability of vitamin D<sub>2</sub> suspected).</p> <p>Not tested in human intervention trial</p> <p>A 5-wk winter-based, single-blinded, RCT [128]: Consumption of UV-exposed mushrooms (containing 491 µg vitamin D<sub>2</sub>/100 g fresh weight) in the form of soup once per week over 4 weeks, increase in mean</p>	<p>Consumer acceptability trial showed sensory parameters similar across treatments</p> <p>Consumer acceptability trial showed sensory parameters similar across treatments</p>

**TABLE 58.5** Overview of foods with increased vitamin D content through bio-fortification, either through animal feed fortification or UVB irradiation.—cont'd

Vitamin D-biofortified foods [Reference(s)] <sup>a</sup> [means of enhancement]	Vitamin D content achievable (µg/100 g) <sup>b</sup>	Evidence of effectiveness	Other information
	provided [125]: Yield range: 0.9–1340 µg/day dry weight Dose range: 0.2–379 J/cm <sup>2</sup> dry weight, various wavelengths and exposure times.	serum 25(OH)D from 37.6 to 59.6 nmol/L. Systematic review and meta-analysis of the impact of UV-exposed mushrooms on serum 25(OH)D response in 6 identified RCTs showed serum 25(OH)D was not significantly increased by UV-exposed mushrooms, but there was high heterogeneity [128].	

<sup>a</sup>Selected exemplars, not intended as a systematic review of the available evidence on each food.

<sup>b</sup>Unless indicated as otherwise.

Modified from Ref. [53].

response of serum 25(OH)D in RCT settings, as mentioned before, dietary modeling analysis based on data from nationally representative dietary surveys within the EC-funded ODIN project (Food-based solutions for optimal vitamin D nutrition and health through the life cycle, <https://cordis.europa.eu/project/id/613977>) has provided in silico projections of how these food interventions may impact on the degree of vitamin D intake inadequacy in the population. For example, a combination of biofortified foods (such as eggs, beef, and pork) together with fortified milk and cheese allowed for projected mean and median intakes close to the EAR to be achieved, without increasing the risk of excessive intakes [106]. In line with these findings, a recent dietary modeling analysis of the impact of vitamin D biofortification of pork and pork products on population vitamin D intakes in the UK [124]. Using four theoretical dietary scenarios, where the vitamin D content was increased 50%, 100%, 150%, and 200% over that present normally, the predicted increases in vitamin D intake in the population were 4.9%, 10.1%, 15.0%, and 19.8%, respectively. However, in quantitative terms this means at the highest increment (200%), the mean vitamin D intake in the population increased from 2.47 to 2.96 µg/day [124].

Finally, biofortification with vitamin D could also embrace the practice of UV irradiation of mushrooms and baker's yeast, which have been shown to stimulate their endogenous vitamin D<sub>2</sub> content. These foods may be a useful strategy to increase vitamin D intakes for vegetarians or those who do not consume meat or foods of animal origin for cultural reasons, but also for the wider population, particularly in the case of bread, as mentioned before and particularly in the context of more flexitarian-type diets of the future. From a technological perspective, UV-irradiated foods can be

produced with anything from low to very high levels of vitamin D<sub>2</sub> [125]. However, in terms of RCT-based evidence of effectiveness, a recent 8-week RCT within the ODIN project [126], investigated the bioavailability of D<sub>2</sub> from UV-irradiated yeast present in bread in healthy 20- to 37-year-old women ( $n = 33$ ) in Helsinki (60°N) during winter. Four study groups were given different study products, either a placebo pill and regular bread (providing 0 µg vitamin D<sub>2</sub> or D<sub>3</sub> per day); a vitamin D<sub>2</sub> supplement and regular bread (providing 25 µg vitamin D<sub>2</sub> per day); a vitamin D<sub>3</sub> supplement and regular bread (providing 25 µg vitamin D<sub>3</sub>/d); or a placebo pill and vitamin D<sub>2</sub>-fortified bread (made with UV yeast) (providing 25 µg D<sub>2</sub>/d). Serum 25(OH)D concentration was measured at baseline, midpoint (week 4), and endpoint (week 8). The study showed that serum 25(OH)D concentrations did not change in those consuming UV yeast—vitamin D<sub>2</sub>-fortified bread only. Those receiving supplemental vitamin D (as either D<sub>3</sub> or D<sub>2</sub>) together with regular bread had appreciable increases [126]. Analysis of the UV yeast bread following baking confirmed that it contained the 25 µg vitamin D<sub>2</sub>, which suggests that vitamin D<sub>2</sub> from UV-irradiated yeast in bread, despite being present postbaking, is not currently bioavailable in humans. EFSA has approved treatment with UV radiation of bread, after the baking process is complete [127]. This allows the ergosterol, which is present in bread as a result of yeast fermentation, to be converted to vitamin D<sub>2</sub>. The bioavailability of the vitamin D<sub>2</sub> from this novel bread has not been examined thus far.

The RCT data demonstrating that the vitamin D<sub>2</sub> in UV-treated mushrooms can increase vitamin D status of consumers have been quite mixed, and accordingly, we undertook a systematic review and metaanalysis of the impact of UV-exposed mushrooms on serum



25(OH)D response in these RCTs [128]. Our structured search yielded six RCTs meeting our inclusion criteria, and a metaanalysis of these six RCTs showed serum 25(OH)D was not significantly increased ( $P = .12$ ) by UV-exposed mushrooms, but there was high heterogeneity ( $I^2 = 87\%$ ). Including only the three European-based RCTs (mean baseline 25(OH)D, 38.6 nmol/L), serum 25(OH)D was increased significantly by UV-exposed mushrooms, whereas there was no significant effect in the 3 US-based RCTs [ $P = .83$ ; mean baseline 25(OH)D: 81.5 nmol/L] [128]. Analysis of serum 25(OH)D<sub>2</sub> and serum 25(OH)D<sub>3</sub> ( $n = 5$  RCTs) revealed a statistically significant increase and decrease after supplementation with UV-exposed mushrooms (weighted mean differences of 20.6 nmol/L and  $-13.3$  nmol/L, respectively:  $P < .001$ ) [128]. Thus, consumption of UV-exposed mushrooms may increase serum 25(OH)D when baseline vitamin D status is low via an increase in 25(OH)D<sub>2</sub> and despite a concomitant but relatively smaller reduction in 25(OH)D<sub>3</sub>. However, when baseline vitamin D status is high, the increase in 25(OH)D<sub>2</sub> and a relatively similar reduction in 25(OH)D<sub>3</sub> explain the lack of effect on serum 25(OH)D. Since this systematic review, two additional RCTs with UV-exposed mushrooms have been published [129,130]. One was an Australian-based, 6-month double-blind, RCT on the effects of supplemental vitamin D<sub>3</sub> (15 µg/day), vitamin D<sub>2</sub> (15 µg/day)-enriched mushroom, standard mushroom, and placebo (all as capsules) on cognition and mood in 436 healthy older adults ( $\geq 60$  years). The study was conducted from April to October (Autumn–Winter) over two successive years. However, compared with placebo ( $-12.2$  nmol/L), the decline in total 25(OH)D in the vitamin D<sub>2</sub>–mushroom group over the 24 weeks of intervention was smaller ( $-7.9$  nmol/L) and slower, while the supplemental vitamin D<sub>3</sub> group increased ( $+4.4$  nmol/L) [129]. This study also showed that in relation to serum 25(OH)D<sub>3</sub>, which declined in the placebo group over the course of the 24 weeks of intervention, it was more pronounced and faster in the vitamin D<sub>2</sub>–mushroom group, and this was evident even when subjects were stratified by baseline total serum 25(OH)D  $< \text{or } \geq 75$  nmol/L [129].

The second RCT was based in the United Kingdom, and in this study, 28 recreationally active, healthy adults were randomized to receive 25-µg encapsulated mushroom-derived vitamin D<sub>2</sub>, matched-dose encapsulated vitamin D<sub>3</sub>, or placebo for 12 weeks (months of the year not reported) [130]. Over the 12 weeks of intervention, the mean total serum 25(OH)D in the group receiving vitamin D<sub>3</sub> capsules increased from 46 to 88 nmol/L, due to increases in serum 25(OH)D<sub>3</sub>, whereas in the placebo group, total serum 25(OH)D remained relatively stable over the period. In contrast, the mushroom–vitamin D<sub>2</sub> group had a significant

increase in mean serum 25(OH)D<sub>2</sub> over the 12 weeks (from 7.0 to 31.4 nmol/L), but serum 25(OH)D<sub>3</sub> decreased from 50.8 to 34.4 nmol/L over the same period [130]. Thus, total 25(OH)D remained unchanged throughout the 12-week period. The FDA has an approved list of foods in which vitamin D<sub>2</sub>–mushroom powder may be used as a source of vitamin D<sub>2</sub> [131].

Artificial UV light technology has also recently been shown to increase the vitamin D<sub>3</sub> content of exposed milk during processing to enhance shelf life, a process approved by EFSA [132]. To our knowledge, no clinical studies have been carried out using this product.

## 6. Safety considerations and prerequisite data required prior to initiating public health measures

Nutrient intake, unlike substances such as drugs or chemical toxicants where there is zero to minimal background exposure, can pose a dual risk, due to either inadequate or excessive consumption. Concentrations of circulating 25(OH)D increase in response to ingestion of vitamin D and reach a steady state after 6–8 weeks [133,134]. If the dose of vitamin D increases, circulating 25(OH)D will continue to rise. High serum 25(OH)D concentrations ( $>220$  nmol/L) may lead to hypercalcemia [135], defined as a serum calcium  $>2.63$ – $2.75$  mmol/L (as defined by individual laboratory reference ranges), and is mainly related to primary hyperparathyroidism or malignancy, but can also be induced by very high calcium or vitamin D intakes. Vitamin D ingested orally is relatively safe, and toxicity is not apparent at doses up to 250 µg/day [14,105]. However, the International Agency for Research on Cancer [136], among others, has pointed out that there are no data on the health hazards of maintaining high serum 25(OH)D in healthy persons over long periods and urged caution to be mindful of past experiences with other compounds (e.g., some antioxidants and hormone replacement therapies) that showed serious adverse effects when chronic high-dose supplements were used. Over and above the risk of hypercalcemia, reports of U- and reverse J-shaped distributions for serum 25(OH)D and adverse consequences, including all-cause mortality, cardiovascular disease risk, PTH suppression, and fetal growth restriction [4,14,137], are being considered on an ongoing basis. Safety data from the ODIN project show that the prevalence of high serum 25(OH)D (i.e.,  $>125$  nmol/L) was only 0.1% for fortified foods compared with 9.4% among the participants who ingested vitamin D supplements [138].

The ULs for vitamin D from the IOM [14] and the EFSA [105,139] were established on the basis of minimizing the risk of hypercalcemia and using evidence from vitamin D supplementation studies. An additional cautionary note was introduced due to the lack of



empirical data on the potential adverse effects of chronic high consumption of vitamin D producing a sustained serum 25(OH)D above  $\sim 125$ – $150$  nmol/L. Thus, to maximize public health protection, an uncertainty factor of 1.2–2 was applied, and both agencies assigned a UL for vitamin D of  $100\text{ }\mu\text{g/day}$  for all adults including pregnant and lactating women [14,105]. The ULs proposed by both organizations were generally similar with slight variation among children. In 2012, the EFSA [105] set a UL for vitamin D of  $25\text{ }\mu\text{g/day}$  for infants up to 1 year;  $50\text{ }\mu\text{g/day}$  from 1 to 10 years, and  $100\text{ }\mu\text{g/day}$  for children aged 11 years and over. The IOM [14] scaled the UL as follows:  $25\text{ }\mu\text{g/day}$  for infants aged 1–6 months, increasing to  $38\text{ }\mu\text{g/day}$  up to 12 months,  $63\text{ }\mu\text{g/day}$  for 1–3 year olds, and  $75\text{ }\mu\text{g/day}$  from 4 to 8 years. The UL for children aged 9 years and over is the same as adults, at  $100\text{ }\mu\text{g/day}$ . Since the IOM and EFSA published their opinions, Gallo et al. [140] reported a randomized controlled dose–response trial in infants in Montreal to test the efficacy of vitamin D at 10, 20, 30, and  $40\text{ }\mu\text{g/day}$  in maintaining 25(OH)D concentrations  $>75$  nmol/L over an 11-month period. By 3 months, 55% of infants in the  $10\text{ }\mu\text{g/day}$  group and 80%–100% of those in the higher doses had a serum 25(OH)D concentration  $\geq 75$  nmol/L. The authors discontinued the  $40\text{ }\mu\text{g/day}$  dose because almost all infants on this dose had serum 25(OH)D concentrations  $\geq 250$  nmol/L by 3 months of age. In addition to valuable information on the nutritional requirement for vitamin D in infancy, these data provided evidence to underpin the lower EFSA UL of  $25\text{ }\mu\text{g/day}$  for infants from 6 to 12 months set in 2012 [105]. Notwithstanding these data, the EFSA revised its UL for infants aged 6–12 months to  $35\text{ }\mu\text{g/day}$  in 2018 [139]. In Europe, the EFSA has recently issued a draft scientific opinion on the Tolerable Upper Intake Level for vitamin D in all age-groups except infants, with the public consultation on this due to close on 5th June 2023 [141]. The proposed ULs for vitamin D in this draft opinion remain unchanged from those set in 2012 (see above).

In terms of monitoring the safety of any dietary strategies to increase vitamin D intakes in the population, the main requirement is that intakes at the 97.5th percentile of the distribution do not exceed the UL for a specified age group. Food-based strategies for vitamin D fortification by nutrient addition or biofortification are designed to increase habitual vitamin D intakes in the population, operate principally on the basis of providing a modest increment in vitamin D across a range of foods to accommodate the diverse dietary practices in modern society. The objective is to change the *shape* of the vitamin D intake distribution to ensure that average and median intakes are at least  $10\text{ }\mu\text{g/day}$ , without increasing the intake at the 97.5th percentile. Data

requirements to assess the risk of exceeding the UL are population-based dietary surveys of representative samples of individuals that include vitamin D intakes from the base diet, from fortified foods and from nutritional supplements. Quality survey data collected on an individual basis, supported by up to date food composition data, can therefore be used in modeling experiments to test the likely effects, within specified age groups, of making changes to the vitamin D supply in the food chain. In particular, modifications to commodity items, such as milk and bread, should have thorough population-based modeling data to support a risk–benefit analysis prior to implementation.

## 7. Conclusions

There is widespread acknowledgment of the presence of very low vitamin D status in the community and the pressing need to address this ongoing problem. Currently, the major regulatory authorities recommend vitamin D intakes in the order of  $10$ – $20\text{ }\mu\text{g/day}$ , depending on age and whether the serum 25(OH)D target is  $\geq 25$  or  $\geq 50$  nmol/L. The current dietary supply of vitamin D makes it unfeasible for the vast majority of children and adults to meet recommended intake targets. Evidence is now overwhelming, from multiple strands including RCTs, systematic reviews, and meta-analyses of RCTs among adults and children across the globe, that vitamin D fortification is effective at increasing vitamin D status. National surveys and case studies of countries that have implemented fortification strategies with appropriate monitoring have demonstrated that targeted approaches can bridge the gap between current and recommended intakes of vitamin D, without increasing the risk of excessive intakes. Traditional food vehicles, such as milk, are only one element of a broader approach to include vitamin D enhancement in a range of products for the benefit of the wider community. There is now trial evidence for the effectiveness and safety of multistrand approaches, as, taken together, small increments in the vitamin D content of commonly consumed foods deliver meaningful increases across the intake distribution. For example, eggs show excellent potential for providing a significant quantity of vitamin D, fortified breads can provide additional vitamin D, and there are emerging data for vitamin D<sub>2</sub> enhancement of mushrooms for vegetarian consumers.

Further research is required on the potential role of foods such as meats, fish, UV-irradiated yeast and breads, and oils, promising potential vehicles that require further work to ensure reproducibility and stability of vitamin D levels achieved. Longer-term community-based intervention studies with standardized approaches to trial design,

ensuring data collection of relevance to public health, e.g., safety, are also required, particularly using combinations of fortified foods among participants of different ages. Wider uptake of reference measurement procedures for analysis of serum 25(OH)D and ongoing food composition analysis for vitamin D and 25(OH)D in foods supported by food-based standard reference materials are also key cornerstones of integrated technological, experimental, and modeling efforts to improve vitamin D intakes and prevent nutritional deficiency. Finally, while scientists have known for many years that fortifying food can prevent vitamin D deficiency, to date, insufficient cooperation between scientists and the public, government agencies, policy-makers, and the industry means that knowledge has not translated to action in most countries. Development and implementation of policy requires more than scientific evidence. New approaches to code-signing nutrition policy frameworks are required for knowledge translation.

## 8. Summary points

- Due to insufficient UVB exposure, low vitamin D status is widespread in the community, affecting many millions of people of all ages.
- It is not feasible for most populations to meet their vitamin D requirement from dietary sources, as vitamin D is low in the food supply.
- For the majority of the population from infancy upward, there are multiple strands of evidence from national nutrition and health surveys to RCTs to show that food fortification represents the most efficient and safest opportunity to increase vitamin D intakes across the population.
- Within specific regulatory contexts, vitamin D<sub>2</sub>, vitamin D<sub>3</sub>, and 25-hydroxyvitamin D can be used as food fortificants, with varying levels of efficacy in terms of their ability to increase serum 25(OH)D in consumers of the fortified, or biofortified foods.
- Multistrand approaches to food fortification should be considered, as, taken together, small increments in the vitamin D content of commonly consumed foods can deliver meaningful increases across the population intake distribution.

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# Bariatric surgery, vitamin D, and bone loss

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## OBJECTIVES

- Recognize the indications for bariatric surgery and the most commonly performed bariatric procedures.
- Understand the effects of bariatric surgery on bone turnover, bone mass, bone microstructure, and fracture risk.
- Discuss potential mechanisms for skeletal effects after bariatric surgery.
- Explain the effects of bariatric surgery on vitamin D status and an approach to vitamin D supplementation.
- Understand the effects of Roux-en-Y gastric bypass and sleeve gastrectomy on intestinal calcium absorption.
- Discuss professional societies' recommendations for the prevention and treatment of bone loss after bariatric surgery.

including diabetes, hypertension, dyslipidemia, osteoarthritis, and sleep apnea. Obesity doubles the medical expenditures of adults relative to those of normal weight, and it increases expenditures on inpatient and outpatient care as well as on prescription drugs [2].

## 1.1 Bariatric surgery is an effective treatment for obesity

It can be challenging to attain significant weight loss with lifestyle modifications such as diet and exercise. Even with increasing pharmacologic agents available for weight management, for many people, the magnitude of weight loss needed to achieve health goals exceeds what can be accomplished with medication. Furthermore, if weight loss goals are achieved, weight maintenance can be difficult, in part due to metabolic adaptations and changes in energy expenditure that predispose to weight regain.

In contrast, bariatric surgery is a highly effective treatment for obesity. On average for all bariatric surgical procedures, 33% of weight or 43 kg is lost [3]. Generally, perioperative complications are low, with a 0.3% overall 30-day mortality rate, despite multiple coexisting conditions in people with severe obesity [4]. Long-term weight loss appears to be maintained after bariatric surgery, with a systematic review of studies of more than 10 years' duration documenting procedure-specific mean percentages of excess weight loss (%EWL) of 46%–74% [5]. Interestingly, those who undergo bariatric surgery may be able to escape the eventual metabolic adaptations seen with conventional weight loss. While these mechanisms are not well understood, changes in the gut and brain after bariatric surgery may affect energy homeostasis and behavior

## 1. Introduction

Obesity is a chronic disease of staggering proportions and consequences. Defined as a body mass index (BMI)  $\geq 30$  kg/m<sup>2</sup> (Table 59.1), obesity stems from a complex combination of genetic, metabolic, environmental, behavior, and social factors. The prevalence of obesity among adults in the United States in 2017–20 was estimated to be 42%, with 9% prevalence of severe obesity (BMI  $\geq 40$  kg/m<sup>2</sup>) [1]. Obesity is associated with increased mortality through its linkage to comorbidities

**TABLE 59.1** Body mass index (BMI) classification according to the centers for disease control and prevention.

	BMI (kg/m <sup>2</sup> )
Underweight	<18.5
Normal weight	18.5–24.9
Overweight	25–29.9
Obesity (class 1)	30–34.9
Obesity (class 2)	35–39.9
Severe obesity (class 3)	≥40

in favor of sustained weight loss [6]. Long-term prospective studies have also demonstrated decreased overall mortality rates compared with matched controls [7]. For these reasons, bariatric surgery worldwide has increased more than threefold since the early 2000s, and in 2013, more than 460,000 procedures were performed [8].

In addition to dramatic and sustained weight loss with a mortality benefit, bariatric surgery can significantly improve or resolve obesity-related comorbidities. One metaanalysis demonstrated rates of diabetes resolution in 77%, improvement in hyperlipidemia in 70%, hypertension resolution in 62%, and obstructive sleep apnea resolution in 86% [3]. The improvement and resolution of diabetes is particularly impressive and can occur quickly and before significant weight loss. For example, both insulin sensitivity and insulin secretion have been reported to improve within days after bariatric surgery [9]. These effects are more pronounced after malabsorptive procedures such as Roux-en-Y gastric bypass and biliopancreatic diversion. The mechanisms are not yet understood but may relate to gut changes and altered secretion of neuroendocrine hormones.

## 1.2 Indications for bariatric surgery

The National Institutes of Health (NIH) Consensus Development Panel recommends that bariatric surgery be considered for carefully selected patients with severe obesity as defined by BMI  $\geq 40$  kg/m<sup>2</sup> or  $\geq 35$  kg/m<sup>2</sup> with major obesity-related comorbid conditions [10]. These include conditions such as cardiopulmonary disease (for example, sleep apnea, obesity-related cardiomyopathy) or severe type 2 diabetes mellitus (T2D). There is a newer consensus that bariatric surgery be considered for those with BMI 30–34.9 kg/m<sup>2</sup> and T2D with inadequate glycemic control despite optimal lifestyle and medical therapy [11]. For adolescents, leading professional societies suggest the consideration of bariatric surgery if BMI  $\geq 40$  kg/m<sup>2</sup> (or 140% of the 95th percentile for age and sex) or  $\geq 35$  kg/m<sup>2</sup> (or 120% of the 95th percentile for age and sex) with

concurrent severe comorbid disease [12,13]. The patient should have careful evaluation by a multidisciplinary team with medical, surgical, psychiatric, and nutritional expertise with lifelong medical surveillance.

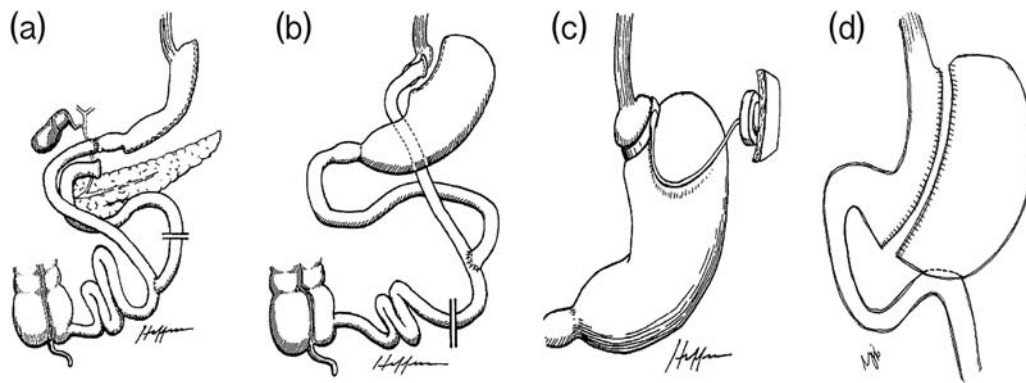
## 1.3 Bariatric surgery procedures

Historically, bariatric surgery procedures were defined as either restrictive or malabsorptive procedures. Restrictive procedures drastically decrease the stomach size to reduce food intake and promote satiety. Malabsorptive procedures decrease intestinal absorption of nutrients by altering intestinal anatomy and changing how bile and pancreatic fluids interact with ingested nutrients. Combination procedures have both a restrictive and malabsorptive component. Additionally, some procedures have a neurohormonal component that influences hunger and energy balance and contributes to weight loss and other metabolic outcomes. Procedures can be performed open or in a laparoscopic manner, although laparoscopic surgery is generally preferred due to lower rates of complications and shorter hospitalizations. The four bariatric procedures most performed to date are reviewed here (Fig. 59.1). There is increasing interest in surgical procedures such as the single-anastomosis duodenoileal bypass with sleeve gastrectomy and the one-anastomosis gastric bypass (“minigastric bypass”), and in endoscopic procedures, but those are not reviewed here due to limited data about their efficacy and role.

### 1.3.1 Roux-en-Y gastric bypass

Roux-en-Y gastric bypass (RYGB), often called “gastric bypass,” has traditionally been considered the gold standard for bariatric surgery due to its effectiveness in inducing weight loss and associated metabolic benefits. Surgeons have been performing gastric bypass for more than 50 years. It was the most commonly performed bariatric procedure in the United States and worldwide until it was overtaken by the sleeve gastrectomy by approximately 2014 [14,15].

RYGB is considered both a restrictive and a malabsorptive procedure, as well as a neurohormonal one. Gastric restriction occurs with the creation of a <30 mL gastric pouch. The malabsorptive element is achieved by dividing the jejunum and creating an anastomosis between the distal jejunum and the gastric pouch. The resulting biliopancreatic limb, which consists of bypassed stomach, duodenum, and proximal jejunum, is anastomosed distal to the gastrojejunostomy. The length of intestine between the gastrojejunostomy and the biliopancreatic limb anastomosis is called the Roux limb, or alimentary limb. The Roux limb is generally 75–150 cm in length.



**FIGURE 59.1** Bariatric surgery procedures. (A) Biliopancreatic diversion with duodenal switch; (B) Roux-en-Y gastric bypass; (C) adjustable gastric banding; (D) vertical sleeve gastrectomy. *Reprinted with permission from Elsevier.*

### 1.3.2 Sleeve gastrectomy

Sleeve gastrectomy is a more recent bariatric surgical procedure that rapidly gained popularity, representing 0% of bariatric operations worldwide in 2003, then 37% in 2013, then overtaking RYGB as the most popular procedure in the United States, and worldwide in approximately 2014 [14,15].

Sleeve gastrectomy is performed by removing >80% of the stomach and creating a tubular section along the lesser curvature of the stomach. The intestinal tract remains unaltered, and so it has been expected to have fewer nutritional deficiencies than a malabsorptive bariatric procedure. Sleeve gastrectomy is less invasive and less technically challenging than RYGB. Interestingly, its mechanism of action is not simply restriction; it also produces neurohormonal effects that induce weight loss. Weight loss is close to that achieved with RYGB [16,17], although resolution of T2D is less than with RYGB [18]. Similar weight loss benefits and lower surgical complication rates have fueled the increasing popularity of sleeve gastrectomy.

### 1.3.3 Adjustable gastric band

Adjustable gastric banding is performed laparoscopically and commonly referred to as the “lap band.” Its popularity peaked in 2008 and then declined. Gastric banding is a reversible restrictive procedure, in which a band is placed 1–2 cm below the gastroesophageal junction to create a gastric pouch <30 mL. The band can be adjusted by injecting saline into a subcutaneous port that is linked to a balloon within the band. The band can be tightened for further weight loss or loosened if the patient has side effects such as nausea or vomiting. There is less long-term weight loss and less improvement in comorbidities than after RYGB [11], and concerns about procedure-specific complications such as band slip/prolapse, esophageal dilatation, band erosion, and device leak have further diminished its use.

### 1.3.4 Biliopancreatic diversion with duodenal switch

Biliopancreatic diversion (BPD) accounts for only a very small proportion of bariatric operations performed [8]. A combination malabsorptive/restrictive procedures with neurohormonal effects, the biliopancreatic diversion induces very substantial malabsorption, with intestinal anatomy altered so that bile and pancreatic fluids do not meet ingested fluids until the ileum. BPD with duodenal switch (BPD-DS), a modification, is now more frequently performed to minimize dumping syndrome, increase gastric restriction, and decrease ulceration. During a BPD-DS procedure, a sleeve gastrectomy is performed, and the surgeon creates a tubular section along the lesser curvature of the stomach. Then a biliopancreatic limb is created and anastomosed to the distal ileum, approximately 50–100 cm from the ileal cecal valve. For intestinal continuity, the ileum is anastomosed to the duodenum. While BPD-DS is one of the most effective procedures for weight loss, it is a technically challenging operation, and there are higher rates of nutritional complications given the greater degree of malabsorption compared with RYGB.

## 2. Skeletal effects of bariatric surgery

The effects of bariatric surgery on biochemical markers of bone turnover and on bone mass are well documented in prospective cohort studies, as summarized in narrative and systematic reviews and metaanalyses [19–24]. The effects of RYGB have been extensively described [25], and fewer studies have addressed other bariatric procedures. Data on the effects of bariatric surgery on bone microstructure are limited for any procedure. These are summarized here, as well as the growing body of literature about fracture risk after bariatric surgery. Emphasis is placed on human studies, but select findings from rodent models of bariatric surgery are



noted as well. To provide additional context for skeletal changes after bariatric surgery, the skeletal effects of nonsurgical weight loss are summarized.

## 2.1 Bone turnover markers

Biochemical markers of bone turnover increase after bariatric surgery [19,26–28]. This phenomenon has been documented after all of the modern bariatric surgical procedures, although the magnitude of bone resorption marker increase appears to vary by procedure. The increases are smaller after gastric banding than after RYGB [29]. RYGB has been shown to produce similar [30] or larger [31,32] increases in bone turnover markers than sleeve gastrectomy.

After RYGB, increases in the bone resorption marker serum C-terminal telopeptide (CTx) are commonly 200% at 12 months [26,27]. In contrast, changes in biochemical markers of bone formation are more variable, with increases usually smaller in magnitude and occasionally not observed at all [26–28]. In one study of 44 adults with obesity undergoing RYGB, an uncoupling index was calculated, reflecting the relative balance of bone resorption and formation [33]. Six-month increases in bone resorption were more than three times greater than increases in bone formation, and there was a positive correlation between the uncoupling index and changes in vertebral volumetric BMD, such that an uncoupling index more heavily favoring resorption was associated with greater decline in vertebral volumetric BMD. “Uncoupling” of resorption and formation has also been reported in rat models of RYGB [34–36].

Increases in bone turnover markers occur very early and are sustained: Serum CTx was recently shown to have increased at a mere 10 days after RYGB [29]. Serum CTx level is significantly higher than baseline by 1 month after sleeve gastrectomy [30] and by 6 months after gastric banding [37]. After RYGB, marker levels peak at 6–12 months but then remain elevated even when weight loss plateaus [38]. Bone turnover marker levels after RYGB and sleeve gastrectomy remain above baseline at least 5 years postoperatively [39,40].

## 2.2 Bone mass

Most clinical studies of the effects of bariatric surgery on bone mass have used dual-energy X-ray absorptiometry (DXA) to assess bone mineral density (BMD). However, assessment of BMD by DXA may be biased in the setting of marked weight loss due to changes in the composition of the soft tissue surrounding bone [41,42]. Further, spurious increases in spinal BMD by DXA may be detected in the setting of degenerative change. Quantitative computed tomography (QCT) is

an established method for assessing volumetric BMD at the axial skeleton or at the appendicular skeleton, the latter sometimes undertaken by high-resolution peripheral QCT (HR-pQCT). Although obesity and weight loss can also influence QCT assessments [43], QCT avoids the traditional biases of DXA. In addition, unlike DXA, these methods can distinguish cortical from trabecular bone compartments. Effects of bariatric surgery on bone mass assessed by DXA are summarized here, along with the data that exist about the effects of RYGB on bone mass assessed by QCT and HR-pQCT.

### 2.2.1 Roux-en-Y gastric bypass

Since the mid-2000s, numerous longitudinal studies have used DXA to evaluate the effects of RYGB on bone mass and have documented clear decreases in areal BMD [21,22,24,25]. At the proximal femur, the magnitude of the decrease in BMD by DXA is striking, with 12-month declines ranging from 5% to 11% [26,27]; on par with the bone mass, a woman might lose over the first 3–4 years of menopause. At the spine, changes in BMD by DXA are variable but usually constitute declines smaller in magnitude than at the hip. Reported changes in BMD by DXA are also variable at the forearm, often with decreases at the ultradistal and total forearm but not the 1/3 distal radius scan regions [26,27].

Decreases in DXA-assessed BMD have been documented as early as 6 months postoperatively [26–28,30,38,44], and progress with time. Longitudinal studies evaluating BMD beyond 1 year after RYGB have shown progressive BMD decline, even after weight loss plateaus [38,45], or despite mild weight regain [46]. This pattern of continued bone loss despite cessation of weight loss has concerning implications for post-RYGB patients.

Because assessment of BMD by DXA may be biased in the setting of marked weight loss, and because spinal BMD by DXA may be spuriously increased in the setting of degenerative change, it has been important to confirm postoperative decreases in bone mass using QCT. Studies examining axial volumetric BMD by QCT or appendicular volumetric BMD by HR-pQCT after RYGB have substantiated the decreases in areal BMD by DXA [25]. DXA may underestimate BMD decline at the spine [25]. For example, in a prospective study of 21 adults followed for 5 years, areal BMD assessed by DXA declined by a mean 7.8% at the spine, while mean decline in trabecular volumetric BMD assessed by QCT was 15.3% [40]. Five-year BMD decline at the hip was similar in magnitude when assessed by DXA and QCT.

### 2.2.2 Sleeve gastrectomy

Because sleeve gastrectomy is a newer procedure, its effects on bone mass have not been defined well.



Human studies to examine bone mass after sleeve gastrectomy are often limited methodologically by lack of prospective design, very short duration, and small size [26,27]. Most have assessed areal BMD by DXA. Studies using DXA have consistently shown decreases in BMD of 3%–10% at the hip over 12 months [47,48]. Changes in BMD at the spine have been inconclusive [24,47], perhaps because the spine is particularly vulnerable to measurement artifact from degenerative disease and soft tissue artifact. QCT may thus be very helpful, although at this time only a few studies exist to have used QCT to measure volumetric BMD after SG [49–51].

Of the studies published to date that compare areal BMD changes after RYGB versus sleeve gastrectomy, several suggest greater BMD reductions at the total hip after RYGB, although others suggest no difference [24]. Two randomized trials comparing RYGB with sleeve gastrectomy, with BMD reported as a secondary outcome, suggest more deleterious loss of bone mass after RYGB than sleeve [52,53]. Meanwhile, in rat models of sleeve gastrectomy and RYGB, bone volume is significantly lower after RYGB than after sleeve gastrectomy [34].

More data are needed about bone mass after sleeve gastrectomy, including data from larger cohorts, CT-based imaging modalities, and longer-term follow-up. With sleeve gastrectomy and RYGB, the predominant procedures performed now, a better understanding of the relative effects of the two procedures will be important for the fully informed decision-making of patients and clinicians alike.

### 2.2.3 Adjustable gastric band

Fewer data exist about bone mass in the gastric banding patient than in the RYGB patient, presumably because use of the purely restrictive gastric band has declined in recent years as interest in studying skeletal outcomes has increased. The published studies have assessed areal BMD by DXA. In general, it appears that BMD decreases modestly at the proximal femur and is preserved at the spine [26,27]. For example, in one longitudinal study of 37 premenopausal women, femoral neck BMD by DXA decreased from baseline by 2.3% at 12 months and by 5.8% at 24 months, while measured spinal BMD had increased from baseline at 12 months and was not significantly different than baseline at 24 months [37]. In a longitudinal study of 26 men and women, total body bone mineral content by DXA declined from baseline by 2.8% over 24 months, on par with a decline of 2.0% observed in a group that underwent medical weight loss [54]. In a study comparing longitudinal changes in BMD by DXA after gastric banding, sleeve gastrectomy, and RYGB, BMD at the proximal femur decreased in all

three groups but more significantly after RYGB than after gastric banding [55].

### 2.2.4 Biliopancreatic diversion with duodenal switch

Consistent with the observations of defective mineralization, increased bone resorption, and decreased bone formation on histomorphometry in an early study of BPD-DS [56], DXA-assessed BMD has been shown to decrease after BPD-DS [57,58]. One longitudinal study reported decreased BMD at the spine after 1 year [57], while another reported no change in BMD at the spine but decreased BMD at the hip after 10 years [58]. As with gastric banding, the paucity of prospective data about bone mass after BPD or BPD-DS is presumably due to the substantial decline in popularity of the procedures.

## 2.3 Bone microstructure

Cortical and trabecular microstructural properties are important determinants of bone quality and strength [59]. Understanding changes in these properties may elucidate the effects of bariatric surgery on skeletal health. In rodents, RYGB has detrimental effects on trabecular [34–36,60] and cortical [36,60] microarchitecture compared with sham surgery. One of the studies examining trabecular microarchitecture showed that rats undergoing sleeve gastrectomy did not experience the detrimental effects [34].

In humans, iliac crest bone biopsies were performed before and 4 years after BPD in 33 adults, with changes including increased osteoid volume and decreased cortical thickness [58]. HR-pQCT is an advanced imaging modality that quantitatively characterizes trabecular and cortical microstructure at the human radius and tibia [61], serving as a “virtual bone biopsy.” Microstructural parameters assessed by HR-pQCT have been reported in several bariatric surgery studies, with participants undergoing RYGB [38,62] or RYGB, sleeve gastrectomy, and gastric banding [63]. Another study looked adolescents and young adults undergoing sleeve gastrectomy, compared with nonsurgical controls [64]. Findings have included deterioration of both trabecular and cortical architecture, with declines in trabecular number and increases in trabecular heterogeneity and decreases in cortical thickness. By finite element analysis, estimated bone strength has been shown to decrease at the radius and/or tibia. In a cross-sectional study of adults who underwent RYGB or adjustable gastric band at least 10 years prior, compared with nonsurgical controls matched on age, sex, and current BMI, those with a history of RYGB had deficits in cortical and trabecular microarchitecture at the radius and tibia compared with controls [65].

## 2.4 Fracture risk

The question of ultimate importance is whether risk of fracture is higher after bariatric surgery, and the literature addressing this question has expanded rapidly, including retrospective and prospective cohort studies [66–76] and systematic reviews and a metaanalysis [24,77]. Interpretation of studies is complicated by heterogeneity in control group selection, but taken together, the literature shows a higher risk of all fractures after bariatric surgery. Fracture risk is time-dependent, as the increased risk seems to appear 2–3 years after surgery [24,77]. For example, in a French population-based cohort study, there were no significant differences in major osteoporotic fracture risk between gastric bypass patients and nonsurgical patients with obesity until 3 years after surgery, at which time fracture risk progressively increased [74].

The increased fracture risk after bariatric surgery is driven by the procedures that have a malabsorptive component, such as RYGB and BPD-DS [24,77]. In a Canadian retrospective cohort study, bariatric surgery in general was associated with an increased risk for fracture, but in procedure-specific analyses, only BPD-DS clearly associated with a higher risk [70]. A Swedish study specifically examined RYGB and documented higher fracture risk than controls with obesity, regardless of diabetes status [73]. Fracture risk does not appear to be increased after adjustable gastric band, and indeed, investigators conducting a pair of studies in the United States used adjustable gastric bypass patients as the comparison group; fracture after RYGB was documented to be higher [71,72].

Whether fracture risk is increased after sleeve gastrectomy remains unclear because it is a newer procedure. Older studies of fracture risk after bariatric surgery included few or no sleeve gastrectomy procedures, and more recent studies including sleeve gastrectomy have shown no increased fracture risk but after limited follow-up time [74,75]. Longer-term follow-up from larger numbers of people undergoing the procedure is needed.

Studies reporting on anatomic site of fracture have yielded variable results. Higher fracture risk has been reported at the hip, wrist, and other upper extremity sites [71–73].

In summary, there is a clear cause for concern that those who undergo weight loss surgery may be at higher risk for fracture-related morbidity and mortality than their peers.

## 2.5 Comparison with nonsurgical weight loss

When considering the clinical importance of bone loss after bariatric surgery and the potential mechanisms of the bone loss, it is worth considering what is known about the skeletal effects of nonsurgical weight loss.

Bone mass does decline with nonsurgical weight loss, and there is interest in weighing whether this is a concerning finding reflective of excessive skeletal fragility, or whether this is simply an appropriate physiologic adaptation to a new, lower body weight [78].

Most studies of bone health during weight loss have examined older adults. Large epidemiologic studies have shown consistently that weight loss is associated with higher fracture risk in older adults [78], although with such studies, there is the possibility that the results are confounded by illness that caused unintentional weight loss and also negative bone effects. To address that concern, older women and men in two large prospective cohort studies were queried about weight loss intention [79,80]. In the setting of overweight or obesity, even voluntary weight loss was associated with incident bone loss and fracture at the hip in older women [79] and incident hip bone loss in men [80]. A number of RCTs, most of 6–12 months' duration, have examined the effects of weight loss interventions on DXA-assessed BMD in older adults with overweight or obesity [78]. In general, the weight loss groups have experienced decreases in body weight of 9%–10% and decreases in BMD of about 2%, with associated increases in bone turnover marker levels.

In contrast to the RCTs of weight loss in older adults, RCTs in younger adults have shown mixed results [78]. Several trials in younger adults have not found BMD decreases by DXA, despite 7%–10% weight loss [81–83]. In a trial of normal weight and mildly overweight adults 20–50 years old who were randomized to caloric restriction or ad lib control diets, the caloric restriction group experienced an 11.5% weight loss over 12 months that was sustained at 24 months (a 10% decrease from baseline) [84]. BMD declined significantly in the caloric restriction group, with an approximately 2% decline by 2 years, and markers of bone resorption increased.

When bone mass is lost during nonsurgical weight loss, it does not seem to be regained in full when weight is regained [85,86]. This is congruent with the observation that when BMD declines and bone microarchitecture changes during spaceflight and with non-weight-bearing after orthopedic surgery, there does not seem to be full recovery after return to normal weight-bearing [87,88].

## 3. Potential mechanisms of bone loss

There are multiple proposed mechanisms for bone loss after bariatric surgery (Table 59.2). Although the specifics and the relative importance of these pathways are unclear, research into potential mechanisms has yielded interesting insights about the relationship between fat and bone, and the roles of muscle, the gut, and the nervous system.

**TABLE 59.2** Potential mechanisms of bone loss after bariatric surgery.

Nutritional	
	Vitamin D deficiency
	Impaired calcium absorption
Mechanical unloading of bone	
Muscle mass loss	
Adipokine changes	
	Leptin
	Adiponectin
Sex steroid changes	
	Estradiol
	Testosterone
Gut hormone changes	
	Peptide YY
	Ghrelin
	Glucagon-like peptide-1
	Glucose-dependent insulinitropic peptide
Bone marrow adipose tissue changes	

### 3.1 Nutritional deficiencies: vitamin D and calcium

Calcium is critical for numerous biological processes, including muscle contraction, neuronal excitability, hormone release, and blood clotting [89]. To facilitate these processes, the body's extracellular calcium concentration is maintained within a narrow range through contributions from the intestine, kidney, and skeleton. Calcium acquisition requires adequate intestinal calcium absorption, and adaptations in renal calcium reabsorption assist with maintenance of homeostasis. The skeleton is a major calcium reservoir, and bone resorption may increase if needed to maintain serum calcium concentration. Calcium homeostasis is regulated primarily by parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}$ ]: A reduction in serum calcium concentration stimulates release of PTH from the parathyroid glands, and PTH enhances renal calcium reabsorption and bone resorption. PTH also stimulates the conversion of 25-hydroxyvitamin D [ $25(\text{OH})\text{D}$ ] to  $1,25(\text{OH})_2\text{D}$ , which then enhances intestinal calcium absorption. In addition,  $1,25(\text{OH})_2\text{D}$  may have direct effects on bone, by increasing resorption and inhibiting mineralization [90].

In the setting of vitamin D deficiency or calcium deficiency (whether from inadequate intake or from

malabsorption), calcium homeostasis is disrupted. When bone resorption increases to maintain the extracellular calcium concentration, it does so at the expense of bone mass and quality, and skeletal health may suffer.

#### 3.1.1 Vitamin D

There is a high prevalence of vitamin D insufficiency or deficiency in the setting of obesity, so those who undergo bariatric surgery may have suboptimal vitamin D status even before their operations. An inverse relationship between BMI and  $25(\text{OH})\text{D}$  level is very well documented [91–93] (See Chapter 75, “Vitamin D, Obesity, and the Metabolic Syndrome,” Hypponen and Boucher.). This has been attributed to sequestration or volumetric dilution of the fat-soluble vitamin in adipose tissue [94,95], and to low sun exposure [96]. Further, obesity has been associated with the decreased expression of enzymes involved in 25-hydroxylation and  $1\alpha$ -hydroxylation in adipose tissue [97], raising the possibility that vitamin D metabolism is impaired in obesity. In studies of individuals with severe obesity preparing for bariatric surgery, mean preoperative  $25(\text{OH})\text{D}$  levels are consistently below 30 ng/mL and often below 20 ng/mL [98].

After RYGB and BPD-DS, problems with vitamin D absorption and intake may threaten vitamin D status. The intestinal absorption of fat-soluble micronutrients, including vitamin D, depends on timely interactions with bile and pancreatic enzymes. Because food and supplements ingested after RYGB or BPD-DS do not mix with bile and pancreatic enzymes until after the biliopancreatic limb joins the alimentary limb to form the common channel, malabsorption of vitamin D may occur postoperatively. In addition, for each of these procedures, because overall food consumption is so dramatically decreased by the restrictive components of the operations, smaller amounts of vitamin D-containing food may be consumed [99].

Indeed, studies have documented a very high prevalence of vitamin D insufficiency or deficiency after RYGB or BPPDS [98,99], although reported prevalence varies substantially due to heterogeneity in postoperative supplementation. After sleeve gastrectomy or adjustable gastric banding, the intestinal, biliary, and pancreatic anatomy remains intact, but postoperative deficiencies nevertheless sometimes occur [98,100–102]. This is likely due to preoperative suboptimal vitamin D status, and further, these procedures restrict postoperative dietary intake, including intake of vitamin D-containing foods. Response to supplementation after bariatric surgery is highly variable [98,99,103], with some individuals achieving  $25(\text{OH})\text{D}$  levels above 30 ng/mL on just 400 IU daily and others requiring 50,000 IU daily or more. Overall, a review of randomized controlled trials

concluded that doses of at least 2000 IU daily are needed to reach 30 ng/mL [104]. Another systematic review and metaanalysis of randomized clinical trials identified that dosages of 2850 IU daily are needed to improve vitamin D status [105]. As discussed in later sections of this chapter, professional organizations recommend preoperative repletion of low 25(OH)D levels, routine postoperative vitamin D supplementation, and monitoring of 25(OH)D levels with the adjustment of supplement dose as necessary [106–109].

### 3.1.2 Calcium

Intestinal calcium transport occurs through both an active, transcellular, saturable pathway and also a passive, paracellular, nonsaturable pathway (See [Chapter 18](#), “Regulation of Intestinal Calcium and Phosphate Absorption,” Fleet and Christakos.). The active, transcellular pathway predominates when calcium intake is low, while passive, paracellular transport increases in importance when calcium intake is high. Active, transcellular calcium transport is primarily regulated by 1,25(OH)<sub>2</sub>D; it is increased in the setting of habitually low calcium intake due to increased PTH secretion and activation of 1,25(OH)<sub>2</sub>D, provided that there is sufficient substrate 25(OH)D [110]. In passive calcium transport, calcium is absorbed in a diffusional manner across tight junctions and intercellular spaces [111]. This paracellular process is nonsaturable and directly related to the concentration of calcium in the intestinal lumen, thereby increasing and achieving particular importance in the setting of high calcium intake. While it was originally thought that paracellular transport was independent of vitamin D, more recent research has revealed regulation by 1,25(OH)<sub>2</sub>D [112]. In particular, 1,25(OH)<sub>2</sub>D appears to induce specific claudin proteins at the tight junctions between cells [113,114].

Active, transcellular transport is prevalent in the proximal small intestine (duodenum and jejunum), was traditionally thought to be absent in the ileum, and occurs to a small extent in the colon, while paracellular transport occurs throughout the length of the intestine. There is evidence, however, that active, transcellular transport occurs in the ileum and that active transport may have greater importance than once thought in the colon [112,115–117]. In transgenic mice with *Vdr* expression exclusively in the ileum, cecum, and colon (and otherwise *Vdr*-knockouts), even just the distal intestinal *Vdr* expression prevented the disrupted calcium metabolism and skeletal phenotype of the *Vdr*-null state [117]. To better understand the mechanisms of intestinal calcium absorption in different segments of the intestine, additional research is needed.

Additional regulators of transcellular calcium transport include estrogen [118], growth hormone [119], and glucocorticoids [120], as well as dietary intake of fat

and fiber [121–123]. In addition, intestinal calcium absorption is impaired in the setting of achlorhydria (decreased gastric acidification) [124].

After RYGB and BPD-DS, one could predict that calcium absorption could be either impaired or normal, based on the normal physiology of intestinal calcium transport and on the altered anatomy after those procedures. On the one hand, calcium absorption could be substantially impaired, due to vitamin D insufficiency or deficiency, or due to the fact that the bypassed duodenum and jejunum, which are usually dominant sites of active, transcellular calcium transport, no longer come into contact with food or supplements. (After RYGB, the proximal jejunum is bypassed; after BPD-DS, the entire jejunum is bypassed). Further, achlorhydria from the operation itself and from the common use of postoperative proton pump inhibitors for marginal ulcer prevention could worsen calcium absorption [125,126]. On the other hand, one could predict that sufficient calcium absorption could be achieved postoperatively if vitamin D status and calcium intake are robust, in light of the evidence for meaningful active, 1,25(OH)<sub>2</sub>D-mediated calcium transport in the distal intestine [116,117], and the passive absorption that occurs throughout the gut.

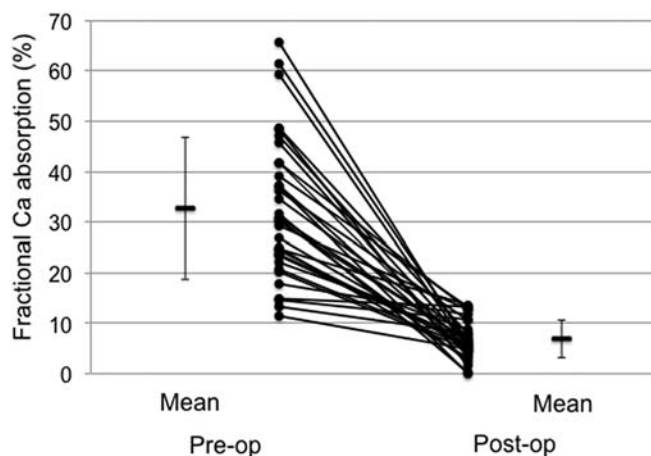
Intestinal fractional calcium absorption (FCA) has been assessed before and after RYGB using dual stable isotopes [127], with one stable calcium isotope administered orally to label dietary calcium and another intravenously to measure calcium removal from the blood. All studies have documented a substantial decline in fractional calcium absorption, although the extent of the decline has ranged from moderate to severe [44,128,129]. One group of researchers measured FCA preoperatively and 6 months postoperatively in 21 women who lost a mean 39 kg during the study period [128]. Mean  $\pm$  standard deviation FCA decreased from 36%  $\pm$  8%–24%  $\pm$  9%. Another group measured FCA in premenopausal women ( $n = 20$  to 23 at each time point) and found that FCA decreased from a mean 41.5%  $\pm$  2.8% preoperatively to 27.9%  $\pm$  3.8% and 18.5%  $\pm$  2.2% at 12- and 24-month time points, respectively [129]. Riedt and colleagues used a larger (200 mg) calcium test load, while Carrasco and colleagues' low-calcium (17.6 mg) test load to measure only active calcium absorption. In both studies, over half the participants had 25(OH)D levels less than 25 ng/mL, and mean postoperative calcium intake was less than 1000 mg at some or all time points. Thus, while these two studies provide very important documentation of FCA decline, it cannot be concluded whether robust vitamin D status and consistent, ample calcium intake could mitigate postoperative declines.

To address that gap in knowledge, in 33 women and men with obesity undergoing RYGB, FCA was



determined in the setting of a target 25(OH)D level of at least 30 ng/mL and calcium intake of 1200 mg daily [44]. Upon enrollment, low 25(OH)D levels were repleted to the target range, and total daily calcium intake was brought to goal through personalized dosing of a calcium citrate supplement, based on dietary intake. Postoperatively, 25(OH)D levels and dietary calcium intake were monitored, and the personalized supplement doses were adjusted as needed to maintain target levels and intakes. Achieved 25(OH)D levels were a median (interquartile range) 41 [33–49] ng/mL preoperatively and 37 [29–40] ng/mL postoperatively. A 10 mg dose of oral calcium-44 was administered in the middle of a standardized test breakfast with 300 mg calcium, and a 3 mg dose of calcium-43 infused intravenously 1 h later, with blood drawn 24 h later for isotope enrichment by mass spectrometry and FCA determination.

Preoperatively, mean FCA was  $33\% \pm 14\%$ , within the range expected for a group composed largely of middle-aged women [121,130]. Postoperatively, FCA decreased dramatically to a mean of  $7\% \pm 4\%$  (Fig. 59.2). Given that total daily calcium intake (from diet and supplements) was 1200 mg at both time points, total daily absorbed calcium fell from  $392 \pm 168$  mg to  $82 \pm 45$  mg. Corroborating and presumably responding to the FCA decline, PTH increased modestly from a median level of 41 [32–53] pg/mL preoperatively to 48 [39–59] pg/mL postoperatively ( $P = .02$ ), and 1,25(OH)<sub>2</sub>D increased from 37 [34–46] pg/mL to 51 [41–63] pg/mL ( $P < .001$ ). Median 24-h urinary calcium decreased ( $P < .001$ ) [44]. Participants with greater percentage weight loss had greater declines in FCA, a finding potentially consistent with the published observation that nonsurgical weight loss results in a decline in FCA compared with weight maintenance [131,132].



**FIGURE 59.2** Fractional calcium absorption before and 6 months after Roux-en-Y gastric bypass surgery, in the setting of standardized calcium intake and robust vitamin D status. Values are mean  $\pm$  SD. Reprinted with permission from Schafer et al. [44].

Alternatively, this finding might be explained by variability in surgical approach, such that those with more intestine bypassed might have experienced more weight loss and also a larger impact on calcium absorption. It is unclear why the observed FCA decrease was more severe than in the studies by Riedt and colleagues and Carrasco and colleagues, but it is clear that maintaining robust vitamin D status and calcium intake does not mitigate the FCA decline after RYGB. Our findings more closely resemble results of early studies of calcium absorption after the jejunoileal bypass, an older bariatric procedure [133,134].

In this cohort of 33 adults undergoing RYGB, those with greater percentage declines in FCA or lower postoperative FCA had greater increases in the bone resorption marker CTx [44]. BMD declined substantially, by a mean 4.6% in areal BMD at the femoral neck and 6.5% in volumetric BMD at the spine. There was not a statistically significant correlation between the extent of the FCA and BMD changes, potentially reflecting the influence of other important factors on BMD, such as mechanical unloading of the skeleton and changes in adipokines and gut-derived hormones, and it does not preclude a contribution of the FCA decline to the BMD decline. In fact, greater increases in PTH—which increased only modestly overall—correlated with greater decreases in BMD at the femoral neck (Fig. 59.2).

A conceptual model for the changes in calcium homeostasis after RYGB integrates these findings (Fig. 59.3). Intestinal calcium absorption is impaired after RYGB, which results in an increase in PTH secretion, which increases bone resorption as a measure to maintain adequate serum calcium concentration. It is possible that 1,25(OH)<sub>2</sub>D has direct skeletal effects as well. Meanwhile, there is concurrent non-PTH-mediated stimulation of bone resorption and loss of bone mass, such that the dramatic increases in bone turnover markers and declines in BMD reflect a multifactorial process. (Such non-PTH-mediated processes are discussed in the next sections of this chapter.) By mobilizing calcium from the skeleton, non-PTH-mediated processes such as mechanical unloading or changes in adipokines or gut-derived hormones may dampen the need for particularly high levels of PTH, providing an explanation for the modest PTH change—or no change—detected in these studies.

Sleeve gastrectomy also results in decreased FCA [129,135]. In one study, FCA was determined using a dual stable isotope approach in premenopausal women ( $n = 13$  to 19 at each time point). FCA decreased from a mean  $36.5\% \pm 2.0\%$  preoperatively to  $21.0\% \pm 2.3\%$  and  $18.8\% \pm 3.4\%$  at 12 and 24 months after surgery, respectively [129]. A study of women and men yielded similar results before and 6 months after surgery: FCA decreased from  $31.4\% \pm 15.4\%$  preoperatively to  $16.1 \pm 12.3\%$  postoperatively, while median



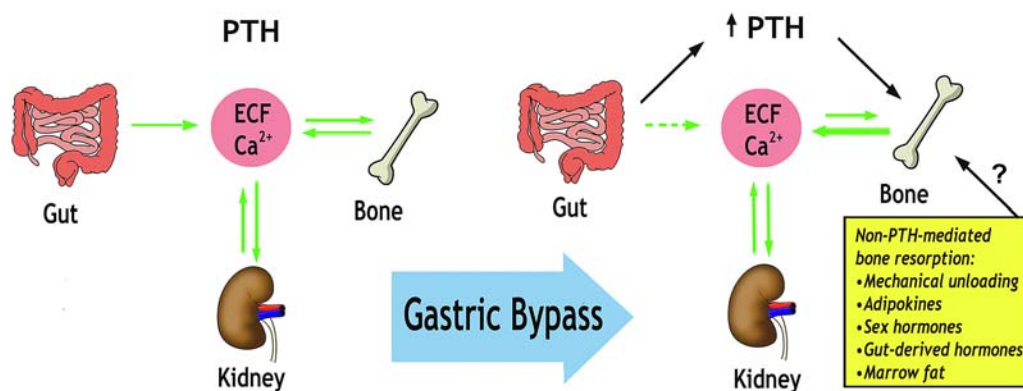


FIGURE 59.3 Effect of Roux-en-Y gastric bypass on calcium homeostasis. Adapted with permission from Elsevier.

(interquartile range) 25(OH)D levels were 39 [32–46] ng/mL and 36 [30–46] ng/mL, respectively [135]. Concurrently, median 1,25(OH)<sub>2</sub>D level increased, without significant changes in PTH level. Individuals with lower postoperative FCA had greater aBMD loss at the total hip.

Why does FCA decline when the intestine remains intact after the sleeve gastrectomy procedure? It is possible that sleeve gastrectomy induces changes in hormones and nutritional parameters implicated in intestinal calcium transport [118,119,121–123]. Indeed, in the 6-month study, participants with greater postoperative increases in IGF-1 levels had higher postoperative FCA [135]. Postoperative use of prophylactic proton pump inhibitors (PPIs) is very common, but a sensitivity analysis was performed excluding PPI users, and the FCA results were similar. Sleeve gastrectomy does diminish gastric acid independent of PPI use, and that may still be a contributor. Further, even nonsurgical weight loss is associated with a decline in calcium absorption capacity compared with weight maintenance [131,132]. Regardless of the reason for the FCA decline, the decline may contribute to loss of bone mass and microstructural integrity after sleeve gastrectomy.

### 3.2 Mechanical unloading

The skeleton adapts to mechanical strain and maintains bone mass and architecture adequate to withstand the loads of everyday activity [136]. Through adaptive remodeling, new bone is added in response to increased loading, and bone is removed in response to decreased loading or disuse. The osteocyte is the major cell type responsible for sensing and translating mechanical strain, as well as orchestrating remodeling [137]. This orchestration is complex and includes the secretion of sclerostin, a load-responsive inhibitor of bone formation [138]. In rodents, skeletal unloading causes loss of bone mass, with

transient increases in bone resorption followed by sustained decreases in bone formation, and with loss of bone mass first at weight-bearing bones [139]. In humans, bed rest [140], space flight [141], and restricted weight-bearing after orthopedic surgery [87] are each associated with declines in bone mass and content. The mechanical unloading has been shown to impact both cortical and trabecular bone mass and microstructure [87,141], with impressive, detrimental increases in the porosity of cortical bone and associated decreases in bone strength.

The dramatic weight loss after bariatric surgery results in the relative unloading of the skeleton. In many studies of bariatric surgery and skeletal health, those with greater weight loss have had greater decreases in BMD [26,27,45]. The hip typically carries more load than the spine, and many studies assessing axial BMD by DXA have shown greater BMD decreases at the hip than spine [27]. Detrimental microstructural changes assessed by HR-pQCT may be worse at the weight-bearing tibia than the non-weight-bearing radius [63]. One recent study showed that sclerostin levels increase after bariatric surgery, and increases in sclerostin correlated with increases in bone turnover markers and decreases in BMD [30].

However, bariatric surgery's effects are complex, and these findings might not simply reflect mechanical unloading. The correlation between extent of weight loss and extent of BMD decline might exist because those with greater weight loss have more severe changes in nutritional status, or more dramatic changes in adipokines, sex hormones, or gut-derived hormones. The correlation could also be the result of measurement bias in the setting of marked weight loss and body composition change. Limitations of available skeletal imaging modalities also require that caution be used when comparing BMD changes at the hip and spine during weight loss [142]. In fact, increases in bone turnover persist [38], and decreases in BMD progress [38,45,46] even after weight loss plateaus. Moreover, a study showed that adults with a history of RYGB at least 10 years prior

had lower BMD and deficits in trabecular and cortical microstructure compared with BMI-matched nonsurgical controls, while adults with a history of adjustable gastric banding were not different than controls [65]. For all these reasons, it seems unlikely that the skeletal changes after bariatric surgery are exclusively the response to mechanical unloading and the appropriate adaptation to a smaller body frame.

### 3.3 Loss of muscle mass

Muscle has long been recognized as the primary source of anabolic mechanical stimuli for bone tissue [143,144]. Skeletal muscle contractions power human body movements, and the long-term loading forces exerted by muscles stimulate increases in bone mass. Age-related losses of muscle mass, strength, and function (termed “sarcopenia”) [145,146] are associated with loss of bone mass, and changes in muscle and bone mass induced by exercise or disuse are usually closely coupled as well. In addition to direct effects on bone, loss of muscle mass, strength, and function can increase fracture risk by increasing fall risk. Contributors to muscle strength and function include diet and hormones such as insulin-like growth factor 1, growth hormone, testosterone, and 1,25(OH)<sub>2</sub>D. Fat infiltration of muscle (myosteatosis) is also a factor [147]. Lipid within and surrounding muscle cells is associated with decreased strength and increased mobility loss in older adults [148,149], as well as with increased risk of incident fracture [150,151].

Bariatric surgery causes loss of muscle mass [152–158], although to a relatively lesser extent than the concomitant loss of fat mass [152,158,159]. Lean mass accounts for an average of 21%–28% of total weight loss in the first postoperative year, with the majority of the muscle mass loss in the first 6 months [152,160]. After the first 6 months, the extent of lean mass lost is highly variable, with some patients experiencing continued loss, and others with muscle mass maintenance or gain [160]. In conjunction with the overall loss of lean mass, absolute muscle strength declines [154,156,158]. However, when muscle strength is considered in relation to body weight (i.e., maximal force or torque per kg body weight), this measure of muscle quality increases [153,156,158]. In muscle biopsy studies, muscle lipid content (intramyocellular lipid) decreases after bariatric surgery [161,162]. Of studies that have examined physical performance before and after bariatric surgery (e.g., with 6-min walk distance), most conclude that physical function improves [153,157,158,163–165].

In the context of dramatic changes in body composition following bariatric surgery, absolute or relative declines in muscle mass and quality might exacerbate decreases in BMD and bone strength, while

improvements in muscle parameters might mitigate the negative skeletal effects of the procedure. Indeed, a correlation between decline in lean mass and decline in BMD has been reported [46,166].

### 3.4 Adipokines

Adipose tissue is more than an energy storage site; it is an active endocrine organ with systemic effects including on bone. There is also cross-talk between adipose tissue and bone. For example, adipocytes secrete several factors known to influence bone physiology such as leptin, adiponectin, tumor necrosis factor- $\alpha$ , and interleukin-6. Meanwhile, bone-derived factors such as osteocalcin and osteoprotegerin directly affect adipocytes, body weight, and glucose homeostasis.

#### 3.4.1 Leptin

Leptin is a hormone secreted by adipocytes that plays an important role in food intake, energy expenditure, reproduction, glucose metabolism, and fat metabolism. Circulation of leptin is proportional to fat stores, so people with obesity have increased plasma leptin levels [167]. Leptin receptors are expressed at high levels in hypothalamic nuclei such as the ventromedial and arcuate nuclei, where leptin serves as a signal of nutritional status and energy storage.

Bone is responsive to energy dynamics and is also affected by leptin. In vitro, leptin appears to have an anabolic effect and has been shown to increase osteoblast proliferation and inhibit osteoclastogenesis, without effect on bone resorption in mature osteoclasts [168,169]. In contrast, in vivo studies of leptin on bone physiology demonstrate a more complex relationship. Leptin-deficient *ob/ob* mice have a high bone mass phenotype, despite hypogonadism and hypercortisolism associated with leptin deficiency [170]. In particular, trabecular volume is increased, whereas cortical bone does not appear to be affected. Leptin is thought primarily to affect bone physiology through neural mechanisms. In mouse genetic studies, deletion of the leptin receptor in neurons results in the high bone mass phenotype seen in leptin-deficient mice, whereas the same deletion in osteoblasts does not influence bone remodeling [171]. Intracerebrovascular infusion of leptin reverses the high bone mass phenotype in *ob/ob* mice and causes trabecular bone loss [170]. Within the central nervous system, sympathetic nervous system signaling appears necessary for the effects of leptin on bone; in mice that have had genetic or pharmacologic ablation of adrenergic signaling, intracerebrovascular infusion of leptin fails to reverse the high bone mass phenotype [172]. Further, there are beta-adrenergic receptors on osteoblasts that regulate

proliferation, and beta-adrenergic agonists decrease bone mass, while beta-adrenergic antagonists increase it [172]. Ultimately, the pathophysiology is not completely understood, and effects of leptin may be different peripherally versus centrally, particularly on cortical bone. For example, *ob/ob* mice that received subcutaneous leptin for 2 weeks had increased tibial bone formation and whole-body bone mineral content [173].

In humans, obesity is associated with higher plasma leptin levels, consistent with leptin resistance, and these levels fall with weight loss [167]. In bariatric surgery, there is a decline in plasma leptin that is associated with the degree of postoperative weight loss [174]. Some studies have identified an association between decrease in plasma leptin and increase in bone turnover marker level or decrease in BMD [45,175], although this association may be confounded by the extent of weight lost.

### 3.4.2 Adiponectin

Adiponectin is a factor secreted by adipocytes and involved in energy homeostasis, glucose, and lipid metabolism as well as inflammation [176]. It is relatively abundant in plasma [177]. Although adiponectin is secreted from adipocytes, levels are paradoxically lower in obesity [177]. Low levels of adiponectin in humans are also associated with insulin resistance and cardiovascular disease.

Most human studies report a negative association between adiponectin and BMD in healthy adults, independent of sex and menopausal status [178]. A relationship between higher adiponectin and lower BMD, independent of fat mass, has also been observed in adolescent girls with anorexia nervosa [179] and in people with diabetes mellitus [180].

In contrast to human studies, findings from in vitro studies are inconsistent, and the mechanism of adiponectin on bone is not completely understood. Both adiponectin and its receptors are expressed from osteoblasts and osteoclasts [181]. Contrary to the negative association with BMD in clinical studies, adiponectin stimulates osteoblasts and inhibits osteoclasts in vitro [182,183]. Adiponectin has also been shown to induce receptor activator of nuclear factor kappa-B ligand (RANKL) and inhibit osteoprotegerin expression in osteoblasts, which contribute to osteoclast formation in a coculture of osteoblasts and peripheral blood monocytes systems [184]. Adiponectin could also affect bone indirectly via its effects on insulin sensitivity or other mechanisms.

Adiponectin levels increase after bariatric surgery [174], consistent with postoperative fat loss. Most studies examining adiponectin level in relation to bone changes after bariatric surgery have not generally suggested an association [45,175,185]. One study that did report a significant association demonstrated that in 42

women undergoing RYGB, adiponectin level at 12 months correlated with total BMD decrease ( $r = 0.35$ ,  $P < .05$ ) after adjustment for baseline weight, baseline body fat, baseline BMD, weight loss, and total and regional body fat loss [159].

## 3.5 Sex steroids

Gonadal steroids are important for skeletal growth and maintenance. In particular, estrogen acts through a variety of mechanisms to suppress bone resorption and increase bone formation. Estrogen plays a significant role in skeletal health in both women and men. In men, testosterone has modest antiresorptive effects and also contributes to bone formation.

Adipose tissue is the main source of estrogen in men and postmenopausal women. In obesity, the excess adipose tissue in men and increased aromatization to estrogen can lead to hypogonadotropic hypoandrogenism. Even though sex hormone-binding globulin (SHBG) is decreased in obesity, free levels of testosterone are low as well.

Bariatric surgery results in dramatic loss of fat mass, which subsequently affects sex hormone levels. These changes have been best described in men, in whom weight loss has been shown to be associated with an increase in total testosterone, SHBG, and calculated free testosterone [186]. Bariatric surgery is more effective at increasing testosterone than low-calorie diet; in a meta-analysis, total testosterone increased on average by 8.73 nmol/L in men undergoing bariatric surgery, compared with an increase of 2.87 nmol/L in men on a low calorie diet [186]. In both groups, those who lost more weight had a further increase in testosterone and gonadotropins and decrease in estradiol. There have been fewer published analyses of sex hormone changes after bariatric surgery in women. One study of 14 premenopausal women and 2 men with obesity showed a decrease in estradiol levels from baseline to 12 months after vertical banded gastroplasty [187]. Although this has not been demonstrated, one might hypothesize that decline in estrogen after bariatric surgery might contribute to bone resorption in men and women, whereas in men, the increase in testosterone may counter that by contributing to bone formation and mitigating bone loss.

## 3.6 Gut-derived hormones

Surgical manipulation of the gastrointestinal tract changes gut physiology, which is thought to play a significant role in the metabolic effects of bariatric surgery. This is postulated because after the gut is surgically altered, the metabolic effects of bariatric surgery are

seen relatively quickly, and before significant weight loss occurs [9]. In addition, feeding is known to decrease bone resorption markers in healthy people, and this phenomenon is preserved and even amplified in those who have bariatric surgery [29,188]. The mechanism for how bariatric surgery changes these gut hormones is not completely understood. Surgically altered nutrient transit is likely an important mechanism; for example, in malabsorptive surgeries, there is increased nutrient delivery to the distal small intestine where there are cells that secrete gut hormones such as peptide YY and glucagon-like peptide-1 (GLP-1). Bariatric surgery in general could also result in enteroplasticity, or multiple gut adaptations driven by the modified environment of the surgically altered gastrointestinal tract, which results in alterations in enteroendocrine cells, the nervous system, gut morphology, and nutrient signaling [189]. Regardless of the mechanism, these gut hormones play significant roles in appetite regulation, energy balance, and glucose homeostasis, and there is increasing evidence of their effects on skeletal physiology.

### 3.6.1 Peptide YY

Peptide YY (PYY) is a member of the neuropeptide Y family and increases in response to food ingestion to promote satiety [190] and to regulate energy homeostasis [191]. Along with other gut hormones such as GLP-1, PYY is produced by L cells in the distal ileum and colon. PYY is a 36-amino-acid peptide that is cleaved by dipeptidyl peptidase IV to form PYY(3–36). PYY(1–36) has high affinity to all four Y-receptor subtypes, whereas PYY(3–36) is a specific Y2 agonist.

Given its role in appetite and energy homeostasis, it is not surprising that PYY levels differ based on body habitus. Those with anorexia nervosa have elevated fasting PYY levels [192], and those with obesity have lower fasting and prandial PYY levels [193]. After bariatric surgery, PYY levels increase [194,195], which may be due to surgical alterations of the gut rather than weight loss. In a study of adults with obesity treated with either RYGB, sleeve gastrectomy, or medical therapy, weight loss at 2 months was similar between groups, but the bariatric surgery groups had an increase in PYY area under the curve (AUC) in response to a meal, while the medical weight loss group had no significant change in PYY AUC [194].

PYY may play a role in skeletal physiology and contribute to bone loss in bariatric surgery. The skeletal effects of PYY may occur via Y receptors: Y2 receptors in the hypothalamus activate the sympathetic nervous system, and peripheral Y1 receptors on osteoblasts inhibit differentiation [196]. Although studies of PYY knockout mice have shown inconsistent results [197,198], human studies suggest a negative correlation between PYY and BMD [199,200]. In the context of bariatric surgery,

in study of RYGB in 44 adults with obesity, 6-month changes in serum PYY correlated with changes in vertebral volumetric BMD changes by QCT and also with the bone formation marker P1NP [33]. In other words, postoperative PYY increases were associated with attenuated increases in P1NP and greater declines in spine volumetric BMD. Consistent with these findings, in a study of adults with obesity and with type 2 diabetes mellitus who had either RYGB or gastric banding, in the RYGB group, there was a positive correlation between changes in fasting PYY and CTx [29]. At 10 days and at 1 year after RYGB, those with a greater increase in PYY had a greater increase in CTx, which suggests that increases in PYY after bariatric surgery could contribute to bone loss following this otherwise beneficial procedure. However, in contrast to these two studies, in a randomized trial of RYGB, SG, or greater curvature plication, there was no relationship between fasting PYY and 1-year BMD change [52].

### 3.6.2 Ghrelin

Ghrelin is an appetite-stimulating hormone produced in the gastric fundus that increases during fasting, decreases after eating, and also reduces energy expenditure. Posttranslationally, ghrelin is acylated with n-octanoic acid or n-decanoic acid, which results in the active acylated form of ghrelin, although the des-acyl form of ghrelin may have biologic activity as well [201]. In humans, ghrelin is increased in anorexia and decreased in obesity [202], which may reflect a compensatory response of ghrelin to these pathologic states. In anorexia, there is reduced feeding; therefore, the observed increase in ghrelin may represent a decreased sensitivity to ghrelin. Conversely, with obesity and the overabundance of feeding, the decreased ghrelin may represent a compensatory adaptation aimed at reducing hunger stimulus.

In humans, the literature describing ghrelin changes after bariatric surgery is heterogeneous, with studies reporting decreases, increases, or no change in circulating levels [203]. Some heterogeneity may be due to measurement, as measured ghrelin level can vary depending on the assay, collection parameters, and whether the total or acylated form is measured. There also appears, however, to be differential effects of various bariatric surgical procedures. The amount of gastric fundus that remains may significantly affect changes in ghrelin levels. In studies of sleeve gastrectomy, where the gastric fundus is resected, total fasting levels of ghrelin have been reported to decrease [204]. In a study of diet-induced weight loss and gastric bypass, diet-induced weight loss of 17% of initial body weight was associated with a 24% increase in the AUC during a 24-h ghrelin profile—consistent with an increase in appetite. In contrast, despite a 36% weight



loss after gastric bypass, the AUC for the ghrelin profile was 77% lower than in normal weight controls and 72% lower in matched controls with obesity. This reflects the appetite decrease that many report after bariatric surgery. After RYGB, there was also a lack of normal meal-related fluctuations and diurnal rhythm of ghrelin levels. This suggests that ghrelin changes after bariatric surgery are unique to the procedure and may contribute to weight-reducing and metabolic effects of surgery.

Ghrelin binds to the growth hormone secretagogue receptor (GHS-R) and therefore may promote bone formation through its effects on growth hormone and insulin-like growth factor-1. There may be a direct effect as well, since osteoblasts secrete both ghrelin and GHS-R, and ghrelin stimulates osteoblastogenesis in vitro [205]. The bone formation effects of ghrelin are also seen in animal models. For example, ghrelin administration to normal and growth hormone-deficient mice results in increased BMD [205]. However, ghrelin knockout mice have the same BMD as controls [206]; therefore, compensatory mechanisms may counteract the effects of ghrelin deficiency. Human studies of ghrelin and BMD have been inconclusive. While there has been a positive association between ghrelin secretion and BMD in healthy adolescent girls [207], other studies have not shown this relationship [178]. Similarly, there is not a consensus in regard to the relationship of ghrelin and bone changes after gastric bypass in the few published studies. One prospective study of women who had either RYGB or sleeve gastrectomy found that 1 year after surgery, decreases in BMD were more dramatic among patients who had greater reductions in ghrelin concentrations [185], but most studies do not report a relationship between a change in fasting ghrelin and skeletal outcomes [33,52,208].

### 3.6.3 Glucagon-like Peptide-1

Glucagon-like peptide-1 (GLP-1) is secreted in the distal ileum and colon and plays an important role in glucose and energy homeostasis. Along with gastric inhibitory peptide (GIP), GLP-1 is an incretin, which is a hormone that is responsible for the phenomenon of enhanced insulin secretion with oral glucose compared with an isoglycemic intravenous glucose load. In addition to stimulating insulin secretion, GLP-1 also slows gastric emptying, attenuates glucagon secretion, and promotes satiety. The United States Food and Drug Administration (FDA) has approved GLP-1 agonists for diabetes treatment as well as weight loss in obesity. After bariatric surgery, most studies show that there is an increase in GLP-1, especially in response to meals. These changes have been observed as early as 1 week after surgery [9] and are influenced by the type of bariatric surgery performed. There is an increase in GLP-1 after procedures with intestinal rearrangement compared

with gastric banding procedures [209], which may be related to an increase in nutrient delivery to the distal ileum where GLP-1 is secreted. However, sleeve gastrectomy also causes an enhanced GLP-1 response to meals [210], which may be due to increased gastric emptying rather than altered anatomical nutritional transit.

GLP-1 appears to have an anabolic effect on bone. GLP-1 may directly influence osteoblastic cells [211] and has been shown to have an insulin-independent anabolic effect in mice after 3 days of a continuous GLP-1 infusion [212]. Similarly, GLP-1 receptor knockout mice have cortical osteopenia, bone fragility, and increased osteoclastic numbers and bone resorption [213]. There have been few studies of bone health in humans with exogenous GLP-1 administration with conflicting results. In one study, participants with type 2 diabetes mellitus on metformin were randomized to either exenatide or insulin glargine for 44 weeks, and after 6% weight loss, there was no difference in BMD [214]. In contrast, in another study, women with obesity who sustained diet-induced 12% weight loss were randomized to liraglutide for 52 weeks, and there was a loss in bone mineral content in the control group, whereas the group that received liraglutide maintained their bone mineral content [215]. In the context of bariatric surgery, the few published studies do not report a relationship between GLP-1 and skeletal outcomes. A study that examined bone turnover markers after RYGB or gastric banding did not find an association between bone turnover changes and fasting or postprandial GLP-1 [29]. Similarly, a randomized control trial of RYGB, SG, or greater curvature plication did not find a relationship between fasting or postprandial GLP-1 with 1-year BMD loss in regression analyses [52].

### 3.6.4 Glucose-dependent insulintropic polypeptide

Glucose-dependent insulintropic polypeptide, also known as gastric inhibitory peptide (GIP), is an incretin that is secreted in the proximal small intestine in the presence of nutrients in the gut. GIP was first named gastric inhibitory peptide because it was thought to decrease gastric acid secretion; however, later studies demonstrated its main effect is stimulating insulin secretion in response to a meal. GIP is also involved in lipid metabolism and is thought to promote fat deposition. Fasting and prandial GIP levels are elevated in obesity and diabetes compared with matched controls [216]. After bariatric surgery, GIP changes appear dependent on the type of surgery performed. In general, the GIP response to a meal is attenuated after malabsorptive surgeries such as RYGB and BPD-DS [217], while postoperative changes in GIP levels are generally observed after restrictive procedures with intact small bowel anatomy, such as gastric banding.



In addition to effects on glucose and lipid metabolism, GIP appears to have an anabolic effect on bone. GIP receptors are located on both osteoblasts and osteoclasts. GIP *in vitro* has an antiapoptotic effect on osteoblastic cells, and it inhibits resorptive activity of mature osteoclasts [218,219]. *In vivo* studies support the concept of GIP as a gut hormone that is anabolic to bone. GIP receptor knockout mice have decreased bone size, lower bone mass, abnormal bone microarchitecture, and impaired biomechanical properties [220]. Similarly, a transgenic mouse model overexpressing GIP demonstrated an increase in bone formation markers, decrease in resorption markers, and increase in bone mass [221]. One GIP receptor knockout mouse model demonstrated an increase in plasma calcium concentrations after meal ingestion, suggesting that GIP may play a role in calcium homeostasis [218].

There have been few studies of GIP and bone in humans. One study found that exogenous administration of GIP did not change serum CTx levels [222]. In contrast, a cohort study found an association between a GIP receptor polymorphism and bone loss. In the Danish Osteoporosis Prevention study, women with a functional GIP receptor polymorphism Glu354Gln (rs1800437) had significantly lower BMD at the femoral neck and total hip, with an increased risk of nonvertebral fractures [223]. There are fewer studies of GIP and bone in the bariatric surgery population. In a study of people with obesity and diabetes who had either RYGB or gastric banding, there was no change in fasting or prandial GIP and no association with bone turnover markers [29].

### 3.7 Bone marrow adipose tissue

Bone marrow fat appears to serve a metabolic role distinct from the role of subcutaneous or visceral fat. Generally, the location of the fat depot in the body affects the function of that fat. For example, excess visceral fat is associated with a greater risk of diabetes, cardiovascular disease, and mortality, in part because visceral fat produces proinflammatory cytokines. Studies of caloric restriction in mice and anorexia in humans have demonstrated that although these are states of lower visceral and subcutaneous fat, there is a paradoxical increase in bone marrow adipose tissue [224,225]. Clearly, marrow fat is acting distinctly from other fat depots, although the physiologic function of marrow fat is not known. Given the proximity to bone, marrow fat may play a role in skeletal physiology. Most of the literature on marrow fat and bone demonstrates a negative association, such that those with higher marrow fat have lower BMD [226]. This has been illustrated in both sexes, at various ages, and in those with and without

osteoporosis [227,228]. Increased marrow fat may exert negative skeletal through multiple mechanisms. First, marrow adipocytes and osteoblasts share a common mesenchymal stem cell precursor within the marrow. Marrow adipogenesis may occur at the expense of osteoblastogenesis. Second, secreted factors from adipocytes, such as adipokines, fatty acids, and cytokines, may negatively affect bone in the local environment.

Interest in marrow fat as a mediator of skeletal health, including after bariatric surgery, has been emerging in recent years. Marrow fat changes after bariatric surgery appear to vary with procedure type. Generally, marrow fat has been reported to be stable or mildly decreased after RYGB [49,51,229–231] and increased after sleeve gastrectomy [49,50]. The mechanisms for procedure-specific differences are unclear, but differences underscore the distinct regulation of marrow fat in the setting of dramatic subcutaneous and visceral fat loss.

Marrow fat studies may also provide insight into the pathophysiology of other metabolic diseases, such as type 2 diabetes, which is associated with an increased risk of fracture [232]. In a study of 30 women with obesity undergoing RYGB, effects of RYGB on longitudinally assessed marrow fat differed by diabetes status, women with diabetes demonstrated a decrease in vertebral marrow fat, whereas women without diabetes had stable marrow fat levels [229]. Changes in hemoglobin A1c and marrow fat were correlated, meaning those who had greater declines in A1c had further decreases in marrow fat. This is consistent with the general finding that type 2 diabetes may be a state of increased marrow fat [233]. Another study did not find marrow fat differences by diabetes status after RYGB [230].

## 4. Prevention and treatment of skeletal effects

### 4.1 Universal recommendations

Professional organizations have issued guidelines for the peri- and postoperative care of the bariatric surgery patient, including those that aim to decrease the risk of skeletal complications of surgery. These references include Clinical Practice Guidelines published by the Endocrine Society in 2010 [106]; Clinical Practice Guidelines issued jointly by the American Association of Clinical Endocrinologists (AACE)/American College of Endocrinology (ACE), The Obesity Society (TOS), the American Society for Metabolic and Bariatric Surgery (ASMBS), the Obesity Medicine Association, and the American Society of Anesthesiologists with most recent update in 2019 [11]; a Position Statement from the ASMBS specifically about metabolic bone changes [234]; and a Position Statement from the European Calcified Tissue Society (ECTS) [24]. Based on the best

**TABLE 59.3** Skeletal health recommendations before and after bariatric surgery.

Before surgery	Check 25(OH)D and replete low levels DXA based on age-appropriate screening Consider DXA in select patients including postmenopausal women and older men
After surgery	
Supplementation	Calcium 1200–1500 mg/day as chewable citrate Consider higher doses for BPD-DS Vitamin D 3000 IU, titrate to >30 ng/mL
Biochemical monitoring	Calcium, albumin, phosphorus, parathyroid hormone, 25(OH)D after 3 months, then every 6–12 months Consider 24-h urinary calcium if additional data needed
BMD monitoring	DXA based on age-appropriate screening; consider in others after 2 years

BMD, bone mineral density; BPD-DS, biliopancreatic diversion with duodenal switch; DXA, dual-energy X-ray absorptiometry.

Modified from AACE/TOS/ASMBS/OMO/ASA, ECTS, and Endocrine Society clinical practice guidelines.

available data, the recommendations are notably limited by a paucity of randomized controlled trials (RCTs) with BMD or fracture outcomes. Our adaptations of these recommendations are listed in [Table 59.3](#).

Preoperative screening of 25(OH)D and PTH levels, with the preoperative treatment of vitamin D and calcium deficiencies, is recommended for patients preparing to undergo any bariatric surgical procedure. Postoperatively, routine supplementation with calcium and vitamin D is also universally recommended. The AACE/TOS/ASMBS/OMO/ASA guidelines specify that calcium supplementation should be with calcium citrate in divided doses, such that elemental calcium intake is 1200–1500 mg/day (from diet plus supplements) for adjustable gastric band, sleeve gastrectomy, and RYGB, whereas supplementation after BPD-DS should include calcium at 1800–2400 mg/day [234]. The same guidelines state that minimum vitamin D intake is 3000 IU daily, as vitamin D<sub>3</sub>, titrated to achieve 25(OH)D levels at least 30 ng/mL. The ECTS suggests calcium 1200–1500 mg/day and vitamin D 400–800 U/day [24].

Postoperative laboratory surveillance recommendations include serum calcium, albumin, 25(OH)D, and PTH levels at a minimum of annually [234]; many also recommend lab surveillance at 3 months and/or 6 months postoperatively. The AACE/TOS/ASMBS/

OMO/ASA guidelines also recommend consideration of measuring 24-h urinary calcium and biochemical markers of bone turnover.

Professional organizations have differed in their recommendations about BMD assessment by DXA, in light of the absence of evidence about the utility of such screening. The Endocrine Society suggests preoperative DXA in all bariatric patients, and DXA yearly until stable after BPD-DS and RYGB, with the consideration of yearly DXA after sleeve gastrectomy and adjustable gastric banding [106]. The ECTS encourages DXA screening in all postmenopausal women and men 50 years old and older before bariatric surgery [24]. The AACE/TOS/ASMBS/OMO/ASA guidelines recommend consideration of DXA at baseline and 2 years post-op in those who have had RYGB or BPD-DS [11]. All bariatric surgery patients should undergo DXA based on age-appropriate recommendations of the Bone Health and Osteoporosis Foundation [235] or the United States Preventive Services Task Force [236].

In addition to these recommended measures, other strategies to consider in the bariatric surgery patient are those that have been shown to attenuate—although not prevent—the loss of the bone mass associated with nonsurgical weight loss in older adults. Exercise, and particularly weight-bearing and muscle-loading exercise, mitigates an increase in bone resorption and decline in BMD when added to caloric restriction [237,238] or when pursued after bariatric surgery [239]. Higher protein intake also attenuates decline in BMD in older adults undergoing caloric restriction [240]. A randomized controlled trial tested the effect of a prebiotic intervention on intestinal fractional calcium absorption in postmenopausal women who previously underwent RYGB [241]. While there was no between-group difference in change in FCA, nor in calciotropic hormones or bone turnover markers, those with greater change in microbial composition following prebiotic treatment had greater increase in FCA, suggesting that manipulation of the gut microbiome may hold potential as an intervention.

An RCT of a multipronged intervention of exercise, calcium, vitamin D, and protein supplementation was shown to attenuate—although not prevent entirely—postoperative increases in bone turnover markers and declines in BMD after RYGB and sleeve gastrectomy [38]. In this trial, the intervention consisted of the combination of 28,000 IU vitamin D<sub>3</sub> weekly for 8 weeks before surgery, 16,000 IU vitamin D<sub>3</sub> weekly with 1000 mg calcium citrate daily after surgery, daily protein supplementation, and daily physical exercise including walking and strength training. The control group received no preoperative vitamin D, no postoperative supplementation with calcium, vitamin D, or

protein, and no obligatory physical exercise. Additional research is needed to identify the relative importance of each of the components of that multipronged intervention.

## 4.2 Management of osteoporosis after bariatric surgery

For those who have had bariatric surgery and are found to be osteoporotic, there is very little evidence to inform management. Antiresorptive agents (bisphosphonates or denosumab) should only be considered in patients after bariatric procedures with osteoporosis once adequate calcium intake and vitamin D status have been confirmed and are stable [11]. This is critical given the elevated risk for bone disease and diminishing supplement adherence with time.

The AACE/TOS/ASMBS/OMO/ASA guidelines caution that if antiresorptive therapy is indicated after bariatric procedures, then intravenously administered bisphosphonates should be used, as concerns exist about adequate oral absorption and potential anastomotic ulceration with orally administered bisphosphonates [11]. If concerns about absorption or potential anastomotic ulceration are obviated, the guideline authors state that oral bisphosphonate administration could be considered. A pilot randomized trial of monthly oral risedronate for the 6 months after surgery showed promise to reduce areal BMD loss after sleeve gastrectomy [242]. However, the ECTS recommends an injectable bisphosphonate as the first-choice treatment and denosumab as second choice [24]. Further research is needed to guide osteoporosis management in this unique patient population.

## 5. Summary points

- Bariatric surgery is an effective treatment for severe obesity. The sleeve gastrectomy and Roux-en-Y gastric bypass are the most commonly performed bariatric procedures.
- After bariatric surgery, there are increased bone turnover, decreased bone mineral density, and diminished bone microstructure. These detrimental skeletal effects are most clearly documented after the Roux-en-Y gastric bypass procedure, with increasing evidence about sleeve gastrectomy.
- Fracture risk increases after the malabsorptive bariatric procedures.
- Potential mechanisms for bone loss after bariatric surgery include nutritional deficiencies; the mechanical unloading of the bone; muscle loss; and

changes in adipokines, sex steroids, gut hormones, and bone marrow adipose tissue.

- Vitamin D deficiency is common after bariatric surgery, and therefore, routine supplementation is recommended.
- Intestinal calcium absorption decreases after Roux-en-Y gastric bypass and sleeve gastrectomy, despite optimization of vitamin D status.
- Professional guidelines offer recommendations about supplementation and skeletal health monitoring, but more supporting evidence is needed to solidify the best clinical practices.

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# Genetic determinants of 25-hydroxyvitamin D concentrations

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## OBJECTIVES

- Present evidence for the role of genetic variation in affecting serum 25(OH)D concentrations.
- Summarize information on variants that have been associated with 25(OH)D concentrations and the possible role these may have in the metabolic vitamin D pathway.
- Discuss practical implications and possible uses of information on genetic determinants of 25(OH)D concentrations.

with estimates as high as 86% reported by some studies [1–4]. However, heritability that can be explained by GWAS (single nucleotide polymorphism–based “SNP” heritability) remains relatively low (around 13%–16%), despite the largest of the studies identifying several independent variants associated with 25(OH)D concentrations [5,6]. In this chapter, we review and describe the key genetic variants associated with 25(OH)D. We focus on genes for which the association has been replicated in independent studies, for many of which we have evidence for a role in the canonical vitamin D pathway.

## 1. Introduction

Vitamin D status (measured by serum concentrations of 25-hydroxyvitamin D, 25(OH)D) is determined by time-of-year and other, largely modifiable, factors that affect either skin synthesis or dietary intake. However, the prevalence of low 25(OH)D concentrations is sometimes high even in sunny locations and in the absence of an obvious behavioral or environmental explanation for the low concentrations. This has accelerated the interest into research on genetic determinants of 25(OH)D, with genome-wide association studies (GWASs) identifying an increasing number of variants. Heritability estimates from twin studies suggest a considerable genetic contribution to the variability in 25(OH)D concentrations,

## 2. 25(OH)D synthesis and metabolism

Most of vitamin D is obtained through skin synthesis catalyzed by ultraviolet B (UVB) radiation exposure, while it can also be obtained through diet or supplements. To obtain the biologically active metabolite 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), vitamin D undergoes two steps of hydroxylation, primarily in the liver and kidney (see Chapter 4 and Chapters 8 and 9). As circulating levels of 1,25(OH)<sub>2</sub>D are under tight homeostatic regulation, 25(OH)D concentrations, which reflect vitamin D intakes during the past 3–4 weeks, are considered as the best measure of vitamin D status [7]. Given the important role of sunlight-induced vitamin D synthesis, 25(OH)D concentrations follow strong seasonal patterns, typically peaking in late summer, and with a trough in late winter/early spring. Indeed, differences in the skin synthesis of vitamin D

are also believed to explain the ethnic differences in skin color. Eumelanin, which provides the pigmentation for darker skins, acts as a natural sunscreen, while more efficient vitamin D synthesis in the lighter skin void of this is likely to reflect an adaptation resulting from migration to less sunny climates during the evolution. As described in Chapter 2, evolutionary genetic adaptations have responded to the dual challenge of protection against high levels of UV radiation and allowing for the photosynthesis of vitamin D. Genes affecting human skin coloration such as *OCA2*, *MC1R*, or *SLC24A4* have, therefore, great interest as candidates for influencing serum 25(OH)D concentrations. However, large-scale GWASs, which have been largely conducted in ethnically homogenous populations (typically white Europeans), did not pick up any of these skin coloration variants as key signals, and even candidate gene studies have often failed to detect related effects. This may be as some of these variants (for example, selected SNPs in *SLC24A5* in Europeans) are so strongly favored that they achieve ~100% frequency in the population [8]. Therefore, while these variants are likely to have a strong effect on vitamin D production, and hence serum 25(OH)D, they will remain undetectable until suitably powered transethnic analyses.

### 3. Heritability of serum 25(OH)D levels

Twin- and family-based studies suggest that genetics contribute substantially to circulating 25(OH)D concentrations [1–4]. Typically, these estimates are derived from comparisons of intraclass correlation (ICC) observed in monozygotic (MZ) twins to that of dizygotic twins (DZ), providing the first impression of the magnitude of the genetic influence. Heritability can also be estimated from unrelated individuals using genome-wide genotyping data (i.e., SNP heritability). However, SNP heritability estimates only capture additive genetic effects, while any nonadditive genetic effects (such as dominance and epistasis) will be neglected [9]. In the largest GWAS analysis to date using data from over 400,000 white Europeans, SNP heritability for serum 25(OH)D concentrations was estimated to be 13%–16%, depending on estimation approach [5,6]. This corresponds to the lowest estimates seen with overall twin heritability, where some studies suggest values up to 86% [10–12]. However, it is important to appreciate that heritability is not a fixed entity, and it depends on the population in which it is estimated and can vary by differences in both genetic background and the environment [13]. For serum 25(OH)D concentrations where UVB radiation dominates as the determinant of circulating concentrations during summer, heritability estimates are strongly affected by season, and 25(OH)D

levels are reported to be highly heritable in winter, with little heritability seen in summer (70% versus ~0%) [11]. Most heritability estimates are based on European ancestry populations, while studies in non-European populations have been limited. A twin study in Hispanics and African Americans, which included data from California, Texas, and Colorado ( $n = 1530$  individuals from 130 families), reported heritabilities of 23%, 28%, and 41%, respectively, for 25(OH)D levels [14]. These differences were broadly reflective of differences in geographical location, such that the higher the latitude (and the less sunlight exposure), the higher the estimated heritability.

To date, there have been only two studies in Asians; one in 109 Chinese twin pairs, which reported a heritability of 69% for 25(OH)D [15], and another one in 1126 Korean male adult twins and family members, which reported a heritability of 51% [16]. These estimates in Asian population were lower than those observed for Caucasians (86%) [12], but higher than those estimated for Hispanics (23%–41%) [14] or African Americans (28%) [14]. These studies may suggest ethnic differences in genetic influences on vitamin D status. However, we cannot exclude the role of differences in the size of the study populations, season, latitude, or other confounding factors, which might explain such ethnic discrepancy. Hence, further heritability studies need to be conducted in ethnically diverse populations living in the same area/latitude and measured the same time of year, to confirm ethnic differences in the heritability of 25(OH)D concentrations.

### 4. Genome-wide association studies of 25(OH)D

GWASs use information across many genomes to test hundreds of thousands of genetic variants to find polymorphisms that are statistically associated with the trait of interest [17]. There are now multiple published GWASs seeking to identify SNPs associated with 25(OH)D [5,6,18–36], although many of these studies have used overlapping samples. Most of the studies only include adult participants of white European ancestry [5,6,20,24,26,30,32]. There are relatively limited data from transethnic analyses [25], and only small studies have been conducted using data from African Americans ( $n = 697$  in the discovery sample) [33], Hispanic ( $n = 229$ ) [19], and Asian populations ( $n = 1387$  to 7590) [23,31,36]. There are also some small GWASs on children/toddlers [21,22,28,29,35], which, however, have largely simply confirmed some of the strongest associations seen in adults.

Three loci, including *DHCR7*, *CYP2R1*, and *GC*, have been consistently reported across European [5,6,18,20,24,26,30,32,34] and several non-European

GWASs [21,23,25,27–29,31,33,35,36]. This is in accordance with the existing evidence of the involvement of their corresponding proteins in vitamin D metabolic pathway. *DHCR7* gene encodes the enzyme 7-dehydrocholesterol reductase, which converts dehydrocholesterol to cholesterol in the skin, thereby affecting the level of substrates required for the synthetic pathway of vitamin D<sub>3</sub>, a precursor of 25(OH)D [37] (Fig. 60.1). Cytochrome P-450 family 2R1, encoded by *CYP2R1* gene, is the key 25-hydroxylase in the liver, which converts vitamin D to 25(OH)D. In the circulation, 25(OH)D is transported by the vitamin D-binding protein (encoded by *GC*) to the kidney, where it is further hydroxylated to form 1,25(OH)D, the functional active form of vitamin D. In addition to these three replicated signals, GWASs conducted on non-European cohorts have also reported several novel loci, which have not been identified in GWASs on white European ancestry. Most of those novel loci have not been independently confirmed, and many have no clear link with vitamin D metabolic pathway, requiring replication in larger samples of respective ethnic groups. Among the suggested novel loci, notable is *CYP2J2*, which was discovered in a multiethnic Asian cohort study consisting of 942 pregnant women of Malay, Indian, and Chinese ancestry [35]. *CYP2J2* encodes the enzyme cytochrome P-450 family 2, subfamily J, and polypeptide 2, which has previously been shown in vitro to hydroxylate

vitamin D [38]. It is potentially interesting that this variant has not been identified in GWASs on white European Ancestry as an influence on 25(OH)D concentrations, a difference that is not explained by frequency of allele distribution (minor allele frequency 29.5% in Asians versus 25.1% in Europeans).

### 5. Variants with evidence for replicated association with 25(OH)D

In the largest-to-date GWAS, loci that affect 25(OH)D concentrations were enriched in genes in the canonical vitamin D metabolic pathway, sulfonation, and glucuronidation pathways (which conjugate sulfur containing molecule and glucuronide to 25(OH)D for excretion and/or recycling), lipid and lipoprotein pathways, and pathways related to skin properties, implicating liver, brain, and skin as the top three locations where 25(OH)D-associated loci may exert their actions [6]. In this section, we provide further details for 35 SNPs that were identified as top hits in this genome-wide association scan in the UK Biobank [6] and that also were associated with serum 25(OH)D concentrations in the SUNLIGHT consortium in a consistent direction [6,26,39] (Table 60.1). These variants are a section of total 143 SNPs associating with 25(OH)D in the UKB GWAS analyses. Variants that could not be replicated in the

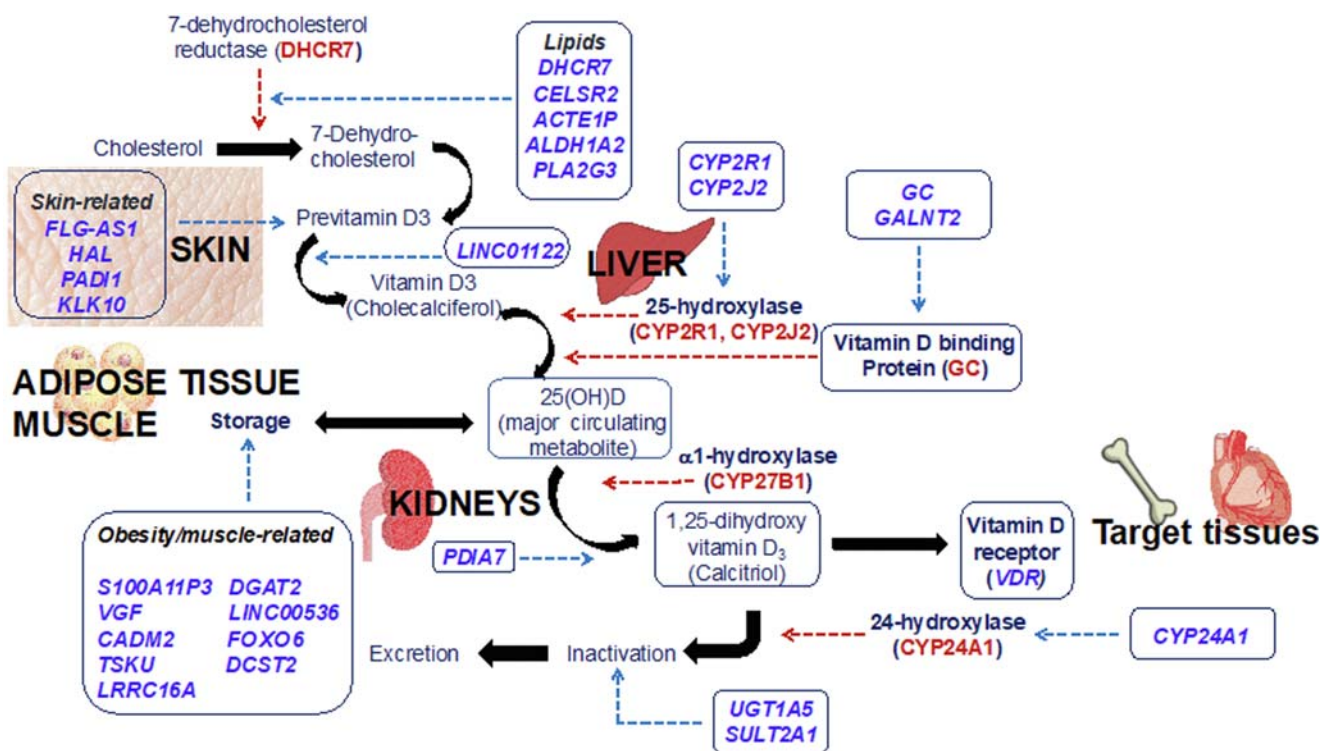


FIGURE 60.1 Possible role of the SNPs in the vitamin D pathway.

**TABLE 60.1** Thirty-five SNPs identified in the genome-wide association scan in the UK Biobank and replicated in the SUNLIGHT consortium.

No.	SNP	CHR	BP	Gene	A1	A2	A1F	UK Biobank <sup>a</sup>			SUNLIGHT consortium <sup>b</sup>		
								Beta	SE	P	Beta	SE	P
1	rs6671730 <sup>c</sup>	1	2339139	PEX10	G	A	0.565714	0.0147881	0.00201077	1.92E-13	0.0061	0.0023	0.006652
2	rs35408430	1	17560195	PADI1	C	T	0.657806	0.0214952	0.00209979	1.36E-24	0.0236985	0.00564768	0.00002715
3	rs7522116	1	41835685	FOXO6	C	T	0.433767	0.0134641	0.00202533	2.97E-11	0.0116727	0.00540416	0.03077654
4	rs7528419	1	109817192	CELSR2	G	A	0.224671	0.0197401	0.00238729	1.35E-16	0.0179046	0.00644566	0.0054732
5	rs1933064	1	152301576	FLG-AS1	A	G	0.46961	0.015731	0.00203195	9.80E-15	0.0155068	0.00539368	0.00404027
6	rs76798800	1	154994978	DCST2	G	T	0.733745	0.0121989	0.00225962	6.71E-08	0.0173898	0.00617041	0.00482841
7	rs6672758	1	230303512	GALNT2	T	C	0.800872	0.0175857	0.00250898	2.40E-12	0.0156301	0.00666121	0.01895423
8	rs727857	2	58981967	LINC01122	G	A	0.388511	0.0140184	0.00206152	1.05E-11	0.0109131	0.00550772	0.04754487
9	rs1047891	2	211540507	CPS1	C	A	0.684179	0.0152142	0.00214041	1.18E-12	0.0126572	0.00572581	0.02706743
10	rs2012736	2	234622379	UGT1A5, ..., UGT1A10 <sup>d</sup>	C	A	0.919186	0.0483073	0.00366555	1.16E-39	0.0384413	0.01038238	0.00021344
11	rs6782190	3	85639672	CADM2	G	A	0.352488	0.0172156	0.00208415	1.45E-16	0.0206718	0.00562451	0.00023756
12	rs705117	4	72608115	GC	C	T	0.1477	0.0334179	0.00280601	1.06E-32	0.0269429	0.00744137	0.00029382
13	rs1352846	4	72617775	GC	A	G	0.708567	0.193471	0.00219074	0	0.2221843	0.00589771	1.40E-310
14	rs78151190	6	25619007	CARMIL1 (LRRC16A)	A	C	0.871284	0.0168754	0.00297406	1.39E-08	0.0187117	0.00829773	0.02413132
15	rs75741381	7	100809458	VGF	C	G	0.852362	0.0166065	0.00282521	4.15E-09	0.0214474	0.00736197	0.00357669
16	rs12056768	8	116988527	LINC00536	T	G	0.417091	0.0234029	0.00202418	6.44E-31	0.0176616	0.00545433	0.00120331
17	rs77532868	10	88081438	GRID1	T	C	0.054042	0.0265692	0.00440069	1.57E-09	0.0280553	0.01353113	0.03813628
18	rs12794714	11	14913575	CYP2R1	G	A	0.578197	0.0878964	0.00201629	0	0.0702488	0.00540376	1.22E-38
19	rs61891388	11	66079818	RP11-867G23.13	G	T	0.455921	0.0125532	0.00200799	4.06E-10	0.0114254	0.00538961	0.03401532
20	rs1660839	11	71094232	AP002387.1	A	G	0.248849	0.0292665	0.00230557	6.40E-37	0.014173	0.00623639	0.02304867
21	rs12803256	11	71132868	AP002387.1	G	A	0.776732	0.104243	0.00239998	0	0.0839119	0.00602549	4.39E-44
22	rs12798050 <sup>e</sup>	11	71223256	S100A11P3	T	C	0.830503	0.109998	0.00264849	0	0.0348	0.0024	1.00E-47
23	rs72997623	11	75488054	DGAT2	A	C	0.084662	0.0276158	0.00358139	1.25E-14	0.0200157	0.00937765	0.03280964
24	rs1149605	11	76485216	RP11-21L23.4	C	T	0.170397	0.0220166	0.00266133	1.31E-16	0.0209786	0.0072426	0.00377288
25	rs10859995	12	96375682	HAL	T	C	0.417366	0.0403465	0.0020206	1.05E-88	0.036551	0.00540543	1.36E-11
26	rs8018720	14	39556185	SEC23A	G	C	0.176673	0.0378247	0.00260904	1.26E-47	0.040852	0.00705183	6.91E-09
27	rs261291	15	58680178	ALDH1A2	T	C	0.644772	0.0273653	0.00208561	2.50E-39	0.0113468	0.0056366	0.04410853
28	rs77924615	16	20392332	PDILT	G	A	0.806515	0.0166321	0.00255158	7.11E-11	0.0195535	0.00670691	0.00355194
29	rs212100	19	48376995	SULT2A1	T	C	0.164001	0.0661522	0.00269018	1.61E-133	0.0193875	0.00719712	0.00706453
30	rs10426	19	51517798	KLK10	A	G	0.213433	0.0256629	0.00243056	4.64E-26	0.0146449	0.0065379	0.02509092
31	rs6123359	20	52714706	BCAS1	G	A	0.102225	0.0341831	0.00331429	6.10E-25	0.0373636	0.00940288	0.00007078
32	rs17216707	20	52732362	CYP24A1	T	C	0.817316	0.0376264	0.00263713	3.47E-46	0.0646902	0.0066412	2.02E-22
33	rs2585442	20	52737123	CYP24A1	G	C	0.240654	0.0356675	0.00237687	6.70E-51	0.0381477	0.00635676	1.96E-09
34	rs2762943	20	52790786	CYP24A1	G	T	0.923071	0.0457231	0.00373798	2.10E-34	0.032534	0.01442103	0.02406994
35	rs2074735	22	31535872	PLA2G3	C	G	0.064096	0.0278196	0.00407045	8.23E-12	0.0213678	0.01054517	0.04273241

<sup>a</sup>Obtained from Revez et al. [6] and used under a CC BY 4.0 License (<https://creativecommons.org/licenses/by/4.0/deed.ast>).<sup>b</sup>Imputed summary statistics, obtained from Revez et al. [6] and used under a CC BY 4.0 License (<https://creativecommons.org/licenses/by/4.0/deed.ast>), serum 25(OH)D has been natural-log transformed.<sup>c</sup>SNP proxy in the SUNLIGHT consortium: rs1123571,  $r^2 = 0.86806$  (1000 Genome, EUR).<sup>d</sup>UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10.<sup>e</sup>SNP proxy in the SUNLIGHT consortium: rs2186777,  $r^2 = 1$  (1000 Genome, EUR).

A1, serum-25(OH)D-increasing allele; A2, alternative allele; A1F, allele frequency for A1; BP, base-pair position; CHR, chromosome number; GRCh37, genome reference consortium human build 37; SNP, single nucleotide polymorphism; SE, standard error.



SUNLIGHT consortium meta-analyses as directly relevant for 25(OH)D concentrations included several that are pleiotropic (i.e., affect multiple traits) and/or are known to affect cholesterol metabolism (e.g., *PCSK9*, *LIPC*, *ABCA1*, *CETP*, *APOE*, *APOB*, *APOC1*, *LIPG*, and *LDLR*). Indeed, one reason that may explain differences between the UK Biobank and SUNLIGHT consortium meta-analysis is that the latter analyses were adjusted for body mass index, which would have prevented some of the adipose tissue-related variants (that may associate with 25(OH)D concentrations through related pathways) from being detected. In the following description and in Fig. 60.1, we describe the possible connections of the replicated variants with vitamin D metabolism. However, despite replicating evidence from two separate sources, SNPs listed in the following may not be causative, and for many cases, the connection, which we have identified with some aspect of vitamin D metabolism/function based on literature available to date, may not fully describe the connection with 25(OH)D concentrations. Therefore, these should be considered primarily as “SNPs of interest,” and where relevant, reflective of possibly interesting target genes for functional studies aiming at characterizing the cellular or physiological contexts of the genetic architecture of 25(OH)D.

**SNP rs6671730:** SNP rs6671730 is an intronic variant (G > A) in the Peroxisomal Biogenesis Factor 10 (*PEX10*) gene (chromosome location: 1p 36.32), which encodes a protein involved in import of peroxisomal matrix proteins. Mutations in *PEX10* gene have led to phenotypes relating to Zellweger syndrome [40], and one of the symptoms of the syndrome includes osteopenia [41], for which vitamin D supplementation has been the treatment. Even though the SNP rs6671730 is an intronic variant, given that alternative splicing has been reported in *PEX10* gene leading to different isoforms of the gene [42], it is possible that the SNP could have a functional impact on *PEX10* gene expression, leading to vitamin D deficiency and resulting in bone-related diseases.

**SNP rs35408430:** SNP rs35408430 is an intronic variant (C > T) in the peptidyl arginine deiminase 1 (*PADI1*) gene (chromosome location: 1p 36.13) that encodes a member of the peptidyl arginine deiminase family of enzymes, which catalyze the posttranslational deimination of proteins by converting arginine residues into citrullines in the presence of calcium ions [43]. As deimination by PADIs is considered a crucial event in epidermal differentiation [44], the connection of the SNP with serum 25(OH)D concentration may be through its influence on skin properties [6].

**SNP rs7522116:** SNP rs7522116 (C > T) is located in the intronic region of the forkhead box O6 (*FOXO6*) gene (chromosome location: 1p 34.2), which encodes a

protein that has been predicted to enable DNA-binding transcription factor activity, RNA polymerase II-specific and RNA polymerase II cis-regulatory region sequence-specific DNA binding activity [45]. It is expressed in the liver, skeletal muscle, and the brain, with abnormal expression associated with metabolic disease, cancer, and altered lifespan [46]. FoxO6 is an important regulator of insulin-stimulated gluconeogenesis in the liver [47], and FoxO6 expression has been demonstrated to be significantly downregulated in the brain of dietary obese mice [48]. Hence, it is possible that the impact of the *FOXO6* on 25(OH)D could have been mediated through its link with obesity and insulin resistance (see Chapter 75).

**SNP rs7528419:** SNP rs7528419 (A > G) is located in the 3'-UTR region of the cadherin EGF LAG seven-pass G-type receptor 2 (*CELSR2*) gene (chromosome location: 1p 13.3), which encodes a protein that has been shown to be a receptor involved in contact-mediated communication, with cadherin domains acting as homophilic binding regions and the EGF-like domains involved in cell adhesion and receptor-ligand interactions [49]. SNP rs12740374 in the *CELSR2* gene has been identified as a causal variant for liver-specific modulation of the expression of *SORT1* (sortilin 1) gene [50], which has been shown to be associated with low-density lipoprotein cholesterol levels and coronary heart disease [51]. Given the biological link between vitamin D and lipid metabolic pathway [52], it is possible that the SNP rs7528419 could act as a proxy for the SNP rs12740374 in the *CELSR2* gene, which may partly explain the association between SNP rs7528419 and 25(OH) D concentration.

**SNP rs1933064:** The SNP rs1933064 (G > A) is an intronic variant in the FLG antisense RNA 1 (*FLG-AS1*) gene (chromosome location: 1q21.3), an RNA gene that is affiliated with the long noncoding RNA (lncRNA) class. Skin pigmentation-related diseases such as ichthyosis vulgaris [53] and peeling skin syndrome 6 [54] have been shown to be associated with *FLG-AS1*. It has been suggested that the SNP rs1933064 may link to serum 25(OH)D concentration through its influence on dermal integrity [6].

**SNP rs76798800:** SNP rs76798800 (G > T) is located in the intronic region of the DC-STAMP domain containing 2 (*DCST2*) gene (chromosomal location: 1q21.3), which encodes a protein product that is predicted to be integral component of membrane. *DCST2* has been identified as an important regulator of osteoclast cell fusion in bone homeostasis [55], which may provide some link to vitamin D [56], but the role of this SNP rs76798800 on vitamin D status is yet to be established. In addition, genetic variant in *DCST2* gene has been shown to be associated with early length and adult height [57], which is suggestive of its possible relationship with vitamin D.



**SNP rs6672758:** SNP rs6672758 (C > T) is an intronic variant in the polypeptide *N*-acetylgalactosaminyltransferase 2 (*GALNT2*) gene (chromosomal location: 1q42.13), which encodes a member of the glycosyltransferase 2 protein family that is involved in O-linked glycosylation of the immunoglobulin A1 hinge region and has been shown to influence triglyceride levels [58] and associated with type 2 diabetes [59], blood pressure [60], and cancer [61]. In addition, the *GALNT2* SNP rs6684432 showed an association with serum vitamin D-binding protein concentrations [62]; even though the *P* value for the association did not reach genome-wide significance, the association could be biologically plausible given that the protein transcripts may be involved in posttranslational modification of vitamin D-binding protein [63], which is likely to affect vitamin D binding, transport, or metabolism. Further molecular studies are warranted to confirm this association.

**SNP rs727857:** SNP rs727857 (G > A) is located in the intronic region of long intergenic nonprotein coding RNA 1122 (*LINC01122*) gene (chromosomal location: 2p16.1), which is an RNA gene that is affiliated with the lncRNA class [64]. A recent study using bioinformatic pathway enrichment analysis has shown that *LINC01122* gene was one of the 989 differentially expressed genes, which was significantly enriched in the vitamin D<sub>3</sub> biosynthesis [65], suggesting the possible biological link between SNP rs727857 and vitamin D status.

**SNP rs1047891:** SNP rs1047891 (C > A) is a missense variant in the carbamoyl-phosphate synthase 1 (*CPS1*) gene (chromosomal location: 2q34). The gene encodes the mitochondrial enzyme that catalyzes synthesis of carbamoyl phosphate from ammonia and bicarbonate [66]. While this SNP rs1047891 is associated with serum 25(OH)D levels with evidence for replication [67], the molecular link between the SNP and vitamin D needs to be established.

**SNP rs2012736:** SNP rs2012736 (C > A) is a missense variant in the UDP glucuronosyltransferase family 1 member A5 (*UGT1A5*) gene (chromosomal location: 2q37.1), which encodes the UDP-glucuronosyltransferase, an enzyme that transforms small lipophilic molecules, such as steroids, bilirubin, hormones, and drugs, into water-soluble, excretable metabolites [68]. Related isoenzymes have been identified as catalysts for 25(OH)D<sub>3</sub> glucuronidation in the human liver, providing a possible link between this SNP and 25(OH)D concentrations [69].

**SNP rs6782190:** SNP rs6782190 (G > A) is an intronic variant in the cell adhesion molecule 2 (*CADM2*) gene (chromosomal location: 3p 12.1), which encodes a member of the synaptic cell adhesion molecule 1 family [70]. Animal studies have established an association between *CADM2* and metabolic traits, including obesity [71],

suggesting that the SNP could influence serum vitamin D concentrations through its effect on obesity and storage capacity of 25(OH)D.

**SNP rs705117:** SNP rs705117 (A > G) is located in the intronic region of the vitamin D-binding protein (*GC*) gene (chromosomal location: 4q13.3), which encodes a protein that binds to vitamin D and its plasma metabolites and transports them to target tissues [72]. Despite being an intronic variant, the SNP rs705117 was identified to be one of the top hits for serum vitamin D-binding protein concentration in the genome-wide association scans [62], suggesting that the SNP could be a proxy for a functional genetic variant.

**SNP rs1352846:** SNP rs1352846 (A > G) is another intronic variant in the *GC* gene. Given that alternative spliced transcript variants encoding different isoforms have been identified for the *GC* gene and intronic variants can impact alternative splicing by interfering with splice site recognition [73], further studies investigating the role of the intronic variants in the alternative splicing of *GC* gene are warranted.

**SNP rs78151190:** SNP rs78151190 (A > C) is an intronic variant located in the capping protein regulator and myosin 1 linker 1 [*CARMIL1* (also called as *LRRCL16*)] gene (chromosomal location: 6p 22.2), which encodes a protein that has been shown to play a role in actin filament network formation, plasma membrane-bounded cell projection organization, and positive regulation of cellular component organization [74]. About 10% of muscle tissue consists of actin, and as alongside with adipose tissue muscle is a key storage site for 25(OH)D [75] (see Chapter 29), this might explain the link with 25(OH)D concentrations.

**SNP rs75741381:** SNP rs75741381 (C > G) is located in the intronic region of the VGF nerve growth factor inducible (*VGF*) gene (chromosomal location: 7q22.1), which encodes a protein that is expressed in neuroendocrine cells and is upregulated by nerve growth factor [76]. *VGF* has been linked with appetite control [77], and animal studies have shown that it is required for diet-induced obesity to develop [78], suggesting a link through variation in the ability to store 25(OH)D.

**SNP rs12056768:** SNP rs12056768 (T > G) is an intronic variant in the long intergenic nonprotein coding RNA 536 (*LINC00536*) gene (chromosomal location: 7q22.1), which is an RNA gene that is affiliated with the lncRNA class. *LINC00536* has been shown to interact with the Wnt3a/β-catenin signaling in the context of its influence on malignant phenotypes of bladder cancer cells [79]. As Wnt/β-catenin signaling is emerging as an important signaling pathway in regulating adipose tissue lipogenesis [80], the association of the SNP rs12056768 with serum 25(OH)D concentration may be mediated through its influence on adipogenesis, affecting the storage of 25(OH)D.

**SNP rs77532868:** SNP rs77532868 (C > T) is located in the intron of glutamate ionotropic receptor delta-type subunit 1 (*GRID1*) gene (chromosomal location: 10q23.1–q23.2), which encodes a subunit of glutamate receptor channels that mediate the fast excitatory synaptic transmission in the central nervous system and play key roles in synaptic plasticity [81]. Animal studies have shown that vitamin D deficiency can accelerate age-related cognitive decline by inhibiting synaptic transmission [82]. Hence, it is possible that the *GRID1* gene could be a target for calcitriol in mediating the synaptic transmission. Functional studies investigating the role of genetic variants in the *GRID1* gene are required to understand the link between *GRID1* and vitamin D status.

**SNP rs12794714:** SNP rs12794714 (G > A) is a synonymous variant in the cytochrome P450 family 2 subfamily R member 1 (*CYP2R1*) gene (chromosomal location: 11p15.2). The gene encodes a member of the cytochrome P450 superfamily of enzymes, which catalyze several reactions involved in the synthesis of cholesterol, steroids, and other lipids. In addition, the enzyme is a vitamin D hydroxylase that converts vitamin D into the 25(OH)D [83]. Several GWASs have identified SNP rs12794714 as one of the top signals for 25(OH)D levels [19,20,26], reflecting the link of this cytochrome P450 enzyme in the synthesis of 25(OH)D.

**SNP rs61891388:** SNP rs61891388 (T > G) has been mapped to the genes, *TMEM151A* and *CD248* (chromosomal location: 11q13.2) [84]. *TMEM151A* has been predicted to be an integral component of membrane, and *CD248* has been shown to enable extracellular matrix binding activity and extracellular matrix protein binding activity in addition to its role in the positive regulation of endothelial cell apoptotic process. Given that active hormonal vitamin D plays an important role in triggering apoptosis in epithelial cancer cells [85], the link between vitamin D and *CD248* is biologically plausible. However, the possible mechanism by which this variant associates with 25(OH)D is yet to be established.

**SNPs rs1660839 and rs12803256:** The SNP rs1660839 (G > A) and rs12803256 (A > G) have been mapped to the gene Actin Epsilon 1, Pseudogene (*ACTE1P*), which is located at chromosome 11q13.4. The gene is an RNA gene and is affiliated with the lncRNA class. The SNP rs12803256 has also been reported as a GWAS signal for vitamin D deficiency in Korean cohorts involving 7590 participants [31]. Both *ACTE1P* [86] and vitamin D [87] have been shown to be involved in the etiology of adolescent idiopathic scoliosis, which is an abnormal curvature of the spine that appears in adolescence, suggesting a possible role of *ACTE1P* in bone health. *ACTE1P* rs12803256 is located in proximity of the *DHCR7* locus (0.01 Mb upstream), and the association

of the SNP with 25(OH)D concentrations may be explained through its links to *DHCR7* function [31].

**SNP rs12798050:** SNP rs12798050 (C > T) has been mapped to the *S100A11* pseudogene 3 (*S100A11P3*) gene (chromosomal location: 11q13.4), which is also called as *S100* calcium-binding protein A11 pseudogene 3. The family members of *S100* proteins have been shown to play multiple roles in buffering calcium ion concentration, participating in energy metabolism, regulating cell proliferation and differentiation, and acting as signaling molecules to activate their corresponding receptors to participate in innate and acquired immune responses [88]. Given the role of vitamin D in calcium absorption [89], energy metabolism [90], cell differentiation [91], and immune system [92], the SNP might have an impact on influencing 25(OH)D levels.

**SNP rs72997623:** SNP rs72997623 (C > A) is an intronic variant in the diacylglycerol O-acyltransferase 2 (*DGAT2*) gene (chromosomal location: 11q13.5), which encodes an enzyme that catalyzes the final reaction in the synthesis of triglycerides in which diacylglycerol is covalently bound to long-chain fatty acyl-CoAs [93]. *DGAT* affects adipose tissue formation [94], and mutations in the *DGAT2* have been shown to be associated with obesity [95], suggesting that it may contribute to 25(OH)D concentrations through influences on storage capacity. Animal studies have also shown that vitamin D receptor upregulates the expression of *DGAT2*, which suggests a coordinated response directed to triglyceride synthesis [96]. This has further been supported by a study that demonstrated an increase in mRNA expression of *DGAT2* in C2C12 myotubes in response to the calcitriol treatment [97].

**SNP rs1149605:** SNP rs1149605 (T > C) has been mapped to the genes, guanylate cyclase 2E, pseudogene (*GUCY2EP*) and tsukushi, small leucine-rich proteoglycan (*TSKU*) (chromosomal location: 11q13.5). *GUCY2EP* encodes a protein involved in chemosensation in rodents, and *TSKU* has been predicted to enable transforming growth factor beta binding activity and act upstream of or within several processes, including negative regulation of Wnt signaling pathway. Even though genetic variations in the Wnt/ $\beta$ -catenin pathway have been shown to be associated with metabolic diseases [98], SNP rs1149605 has not been associated with traits other than 25(OH)D concentrations in GWAS conducted to date; however, other variants in *GUCY2EP/TSKU* have been associated with wide range of metabolic and cognitive traits [99].

**SNP rs10859995:** SNP rs10859995 (T > C) is an intronic variant located in the histidine ammonia-lyase (*HAL*) gene (chromosomal location: 12q23.1), which is expressed in the skin, and is upregulated during the differentiation of keratinocytes [100]. *HAL* deaminates L-histidine to trans-uronic acid [101], which in the stratum

corneum has been shown to absorb UVB [102] and reduce the production 25(OH)D [103]. Therefore, the connection of the SNP rs10859995 with serum 25(OH)D concentration may be through its influence on skin properties [6]. In the analysis of the genotype tissue expression project datasets, one of the *HAL* SNPs, rs17676826, was shown to be significantly associated with its mRNA expression levels in sun-exposed skin of the lower leg [104] suggesting a possible role of *HAL* in influencing 25(OH)D levels in response to sun-light exposure.

**SNP rs8018720:** SNP rs8018720 (G > C) is a missense polymorphism in the Sec23 homolog A, coat complex II component (*SEC23A*) gene (chromosomal location: 14q21.1), which encodes a protein that plays a role in the ER–Golgi protein trafficking. Aside of a replicated association between this SNP and serum 25(OH)D concentration [26,31], rs8018720 has not been associated with other traits [105]. Functional links with vitamin D metabolism are yet to be established.

**SNP rs261291:** SNP rs261291 (T > C) has been mapped to aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*) gene (chromosomal location: 15q21.3), which encodes an enzyme that catalyzes the synthesis of retinoic acid (RA) from retinaldehyde [106]. In addition to 25(OH)D, rs261291 has been associated with multiple lipid related traits in GWASs [107]. The exact role of rs261291 explaining the associations with 25(OH)D and lipid/cholesterol metabolism is yet to be established by functional studies.

**SNP rs77924615:** SNP rs77924615 (G > A) is an intronic variant in the protein disulfide isomerase like, testis expressed (*PDILT*—also known as *PDIA7*) gene (chromosomal location: 16p12.3), which encodes a member of the disulfide isomerase family of endoplasmic reticulum proteins that catalyze protein folding and thiol–disulfide interchange reactions [108]. In addition to 25(OH)D, GWASs suggest a link between rs77924615 with multiple renal traits (kidney stones, glomerular filtration rate, serum creatine, urate concentrations) [109–111], which may suggest a link through kidney function and 1,25-hydroxylation.

**SNP rs212100:** SNP rs212100 (T > C) is an intronic variant in the sulfotransferase family 2A member 1 (*SULT2A1*) gene (chromosomal location: 19q13.33). *SULT2A1* encodes a liver- and intestine-expressed sulfo-conjugating enzyme that is responsible for the inactivation by sulfonation of 25(OH)D [112,113], which is likely the underlying mechanism connecting this SNP with serum 25(OH)D concentration [6].

**SNP rs10426:** SNP rs10426 (G > A) is a 3' untranslated region variant in the kallikrein-related peptidase 10 (*KLK10*) gene (chromosomal location: 19q13.41), which encodes a protein that has been shown to play a role in dermal integrity [114]. It has been suggested

that the connection of the SNP with serum 25(OH)D concentration is likely through its influence on skin properties [6].

**SNPs rs6123359, rs17216707, rs2585442, and rs2762943:** The three SNPs, rs6123359 (A > G), rs17216707 (T > C), and rs2585442 (C > G), have been mapped to the genes, cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) and brain-enriched myelin-associated protein 1 (*BCAS1*), whereas the SNP rs2762943 (T > G) has been mapped to *CYP24A1* and prefoldin subunit 4 (*PFDN4*) genes (chromosomal location: 20q13.2). *CYP24A1* is an important candidate for vitamin D metabolic pathway given that the protein encoded by the gene initiates the degradation of 1,25-dihydroxyvitamin D<sub>3</sub> (i.e., physiologically active form of vitamin D<sub>3</sub>) by hydroxylation of the side chain [115]. In addition, this enzyme also plays a role in calcium homeostasis and vitamin D endocrine system [116]. Furthermore, *BCAS1* and *PFDN4* are some of the target genes for 1,25(OH)2D<sub>3</sub> in cancer cells [117,118], suggesting the possible molecular relationship between vitamin D and the target genes.

**SNP rs2074735:** SNP rs2074735 (G > C) is a missense polymorphism in the phospholipase A2 group III (*PLA2G3*) gene (chromosomal location: 20q13.2), which encodes an enzyme that functions in lipid metabolism and catalyzes the calcium-dependent hydrolysis of the sn-2 acyl bond of phospholipids to release arachidonic acid and lysophospholipids [119]. The mechanism explaining the link between SNP rs261291 and 25(OH)D is yet to be established by functional studies.

## 6. Gene–environment interaction

Gene–environment interaction (GxE) describes the phenomenon where the genetic influences of the traits are modulated by behavior or environment (or vice versa) [120]. At cellular level, this type of effect modification may be explained through epigenetic effects, such as DNA methylation, histone acetylation, and non-coding RNA expression [121], which regulate whether, or the extent to which, the effects of a gene affect the trait of interest. A recent GWAS identified 25 independent loci suggestive of GxE interaction, where the magnitude of the genetic association with 25(OH)D varied by environmental factors influencing serum 25(OH)D concentrations. Of these putative GxE loci, 23 loci were marginally associated with serum 25(OH)D concentration at the genome-wide significant level, and five loci (including *CYP2R1* and *SEC23A* and others) showed genome-wide significant interaction with season [5,6]. Interestingly, at the *CYP2R1* and *SEC23A* loci, serum 25(OH)D concentrations for the carriers of 25(OH)D-lowering allele were less responsive to season compared



with noncarriers, suggesting that some individuals may have steadily low serum 25(OH)D levels regardless of the season of measurement [5]. Further, in an earlier genome-wide GxE analysis, carriers of 25(OH)D-lowering allele at the *CYP2R1* locus also appear to be less responsive to dietary vitamin D intake [26]. A similar GxE interaction pattern has also been observed at the *GC* locus in candidate GxE interaction studies with respect to vitamin D supplementation [122], consumption of vitamin D<sub>3</sub>-fortified bread and milk [123,124] and UVB treatment [123,124]. The extent to which these GxE patterns might have practical implications, for example, by warranting more personalized approaches to vitamin D supplementation, is uncertain (as also discussed in the following).

## 7. Applications and practical implications

Findings from existing GWAS for serum 25(OH)D concentrations have highlighted genes in the canonical vitamin D metabolic pathway. These have also shown enrichment of associations near genes involved in skin synthesis, hepatic hydroxylation, sulfonation, and glucuronylation, suggesting serum levels of 25(OH)D are in cross-talk with a range of related metabolic processes [5,6]. The understanding about genetic loci associated with serum 25(OH)D concentrations has already proven their uses as instruments for studies examining the causal effect of vitamin D on other traits. Sitting at the interface between observational studies and interventional trials, Mendelian randomization (MR) has been increasingly used to strengthen causal evidence in observational studies (see Chapter 61). This approach uses genetic variants associated with the exposure of interest (so-called genetic instrument) to approximate the exposure, and conditional on the key method assumptions being met, MR has the benefit of reducing bias due to confounding and reverse causation [125]. The genetic instrument is normally selected from the genome-wide association analysis of the exposure of interest, often including all independent and genome-wide significant loci. Earlier MR analyses restricting to variants in the canonical vitamin D pathway (including *DHCR7*, *CYP2R1*, *GC*, and *CYP24A1*) have provided insight into the causal nature of the association of 25(OH)D with risks for multiple sclerosis [126], type 2 diabetes [127], and hypertension [128]. With additional loci being discovered for serum 25(OH)D [5,6], many MR studies now incorporate these new loci in the analyses. While inclusion of additional loci can bolster statistical power, it is also important to keep in mind the potential for pleiotropic effects that these variants could bring into the analysis and that could bias the MR analysis [129]. As alluded earlier in the chapter, many newly

discovered variants have less clear/unknown function related to vitamin D metabolism and are strongly related to other traits, such as BMI, cholesterol, and blood pressure, among others. Restricting analyses to variants with consistent replicating associations with 25(OH)D concentrations will help to alleviate related concerns, at least to some extent. To address potential pleiotropy, studies will need to include sensitivity analyses using different sets of variants and different analytical approaches that will help to assess the robustness of their findings. The multivariable MR analyses, which directly account for pleiotropic effects by simultaneously modeling the genetic effects on 25(OH)D and on pleiotropy-related indicators, may also be helpful. However, this is only possible if the relevant pleiotropic pathways can be hypothesized and if relevant information is available for the analyses. Rigorously conducted MR studies taking account of nonlinearity are likely to be of increasing value in the context of vitamin D research, given that like many (most) nutrients and biomarkers circulating concentrations are likely to have threshold effects, where harm is both seen at very low or high levels [130,131]. This is of great value, considering the challenge of recruiting vitamin D-deficient individuals to clinical trials to investigate the health effects of vitamin D supplementation [130,131]. Indeed, recent studies using the nonlinear or stratified MR approach suggest that vitamin D deficiency may be associated with an increased risk for CVD and all-cause mortality, both of which are not evident using the standard linear MR approach [39,132], although evidence for a beneficial role of vitamin D supplementation on mortality has been seen in RCTs [139]. Recently, this approach has also confirmed a role of vitamin D deficiency on dementia risk [133], an association which has also been reported by linear MR studies [134]. However, it is very plausible that many of the benefits may only be seen with the prevention and treatment of vitamin D deficiency, while much of the time, benefits are unlikely for further increases in concentrations for individuals who are already vitamin D replete.

Genetic correlation is another widely used genetic epidemiology approach. This allows us to quantify overlaps in genetic architecture between two traits, and it can provide a better understanding of the shared biological pathways and/or causal relationship between 25(OH)D and other traits [135]. Indeed, genetic correlation analyses using the UK Biobank vitamin D GWAS results across over 700 traits [5,6] have, for example, highlighted that serum 25(OH)D is negatively correlated with cognition-related traits, including time of computer use, educational attainment, and intelligence. Given the potential link between intelligence and years of education, and working indoors [137], this finding is likely reflective of behaviors associated with decreased level of sun exposure [6].

Genetic variants can be combined into a genetic risk score (GRS) to reflect individuals' lifetime genetic risk of disease, with an expectation that GRS may be used to predict disease risk. In the context of serum 25(OH)D, it is reasonable to expect that individuals who carry many 25(OH)D-lowering alleles as reflected by their GRS are more susceptible to a low vitamin D status. In the UK Biobank, the difference in the mean 25(OH)D between individuals in the highest versus lowest quartile of the GRS is about 9 nmol/L, if estimated based on the 35 replicated vitamin D GWAS variants [39]. The magnitude of the association is similar to that seen with self-reported use of vitamin D supplementation in the UK Biobank during winter (9.7 nmol/L) [138] and suggests that when supply from sunlight or diet is limited, people with high genetic burden will be more likely to have lower concentrations that could have clinical relevance. As noted before (see Gene–environment interaction), individuals with genetically low 25(OH)D may also be less responsive to treatments for correcting low vitamin D status [122–124]. For example, in a study using a GRS combining 2 SNPs at *CYP2R1* and *GC* loci, a ~23% increase in serum 25(OH)D after UVB treatments is seen for individuals carrying four risk alleles in contrast to a 54% increase for those carrying no risk alleles [123]. Similarly, individuals carrying four risk alleles also benefitted the least from the 6-month consumption of vitamin D<sub>3</sub>–fortified bread and milk [123]. It has also been suggested that GRS for 25(OH)D may be useful for guiding the screening and treatment for vitamin D deficiency, where individuals with a high GRS score may need to be monitored more often for serum 25(OH)D concentration, and if a low vitamin D status is detected, they may need a higher dose of vitamin D supplementation to achieve the target level. In a recent study [34], participants with serum 25(OH)D < 50 nmol/L received a tailored vitamin D supplementation recommendation based on their genetic risk. Also this study used a simple GRS consisting of 2 SNPs, one from *GC* and another from the *CYP2R1* loci, and the researchers recommended individuals with three or four 25(OH)D-lowering alleles to take 50 µg (2000IU) per day, those with 1 to 2 risk alleles to take 20–30 µg/day and those without any risk alleles 10–20 µg per day. Their GRS-based analyses suggested that recommendation to take 50 µg (2000IU) per day over 4 months was enough to reduce the gap in serum 25(OH)D concentration and prevalence of 25(OH)D < 50 nmol/L between individuals carrying three or four risk alleles and those with no risk alleles (Fig. 60.2). However, the prevalence of 25(OH)D < 50 nmol/L remained elevated for those with 2 risk alleles compared with no risk alleles. While interesting

and very promising, these results remain tentative given the increases in vitamin D intakes were achieved by recommendations, rather than placebo-controlled, blinded, and randomized provision of supplementation. The number of individuals in each of the treatment groups was also small ( $n = 10$  to  $n = 36$ ), and further trials with a larger sample are required to confirm the need, doses, and effective approaches for personalized vitamin D supplementation.

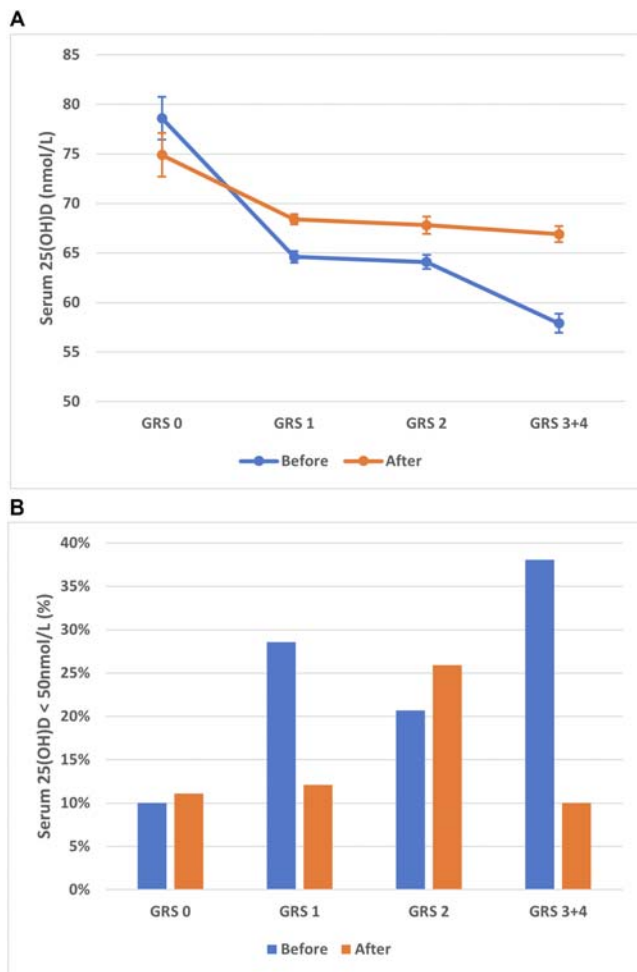
## 8. Conclusions

Genetic variants affect 25(OH)D concentrations, with heritability estimates varying depending on population and time of year. Large-scale GWASs have identified a large number of potentially interesting signals, which, in addition to the canonical vitamin D metabolism pathway, have been linked with various aspects of lipid metabolism, and pathways reflecting skin properties. These variants have provided their uses as instruments to provide genetic evidence for a causal effect of 25(OH)D, with recent applications extending these studies to investigations reflecting expected effects in the treatment of vitamin D deficiency. Further research is required to establish the benefits and uses of genetic information in guiding the need for genetically tailored personalized approaches to vitamin D supplementation and the prevention of vitamin D deficiency.

## 9. Summary points

- Twin studies suggest a considerable genetic contribution to 25(OH)D concentrations, while proportion of variability that can be explained by SNPs remains relatively low (around 13%–16%).
- There are at least 35 independent variants that have been shown to have a replicating and directionally consistent association with 25(OH)D concentrations, with variants in/near the *DHCR7*, *CYP2R1*, *GC*, and *CYP24A1* genes showing consistent associations in various population groups.
- Loci that affect 25(OH)D concentrations are largely enriched in genes in the canonical vitamin D metabolic pathway, lipid and lipoprotein pathways, and pathways related to skin properties.
- Clinical relevance of variants affecting 25(OH)D concentrations remains tentative, and while some pilot data suggests genetic differences in response to vitamin D intakes, further evidence is required to confirm the need and benefits of personalized approaches.





**FIGURE 60.2** Profile of serum 25(OH)D (A) and prevalence of serum 25(OH)D < 50 nmol/L (B) by GRS for serum 25(OH)D before and after GRS-based vitamin D supplementation reported by Sallinen et al. 2021 [34]. Genetic risk score (GRS) for serum 25(OH)D (range from 0 to 4) was calculated as the sum of the number of risk alleles for the SNPs rs4588 (A allele) in the GC locus and rs10741657 (G allele) in the *CYP2R1* locus. Participants with serum 25(OH)D < 50 nmol/L received a tailored vitamin D supplementation recommendation based on their GRS: Individuals with GRS<sub>3+4</sub> was recommended to take 50 µg (2000IU) per day, those with GRS<sub>1</sub> or GRS<sub>2</sub> to take 20–30 µg/day, and those with GRS<sub>0</sub> to take 10–20 µg per day.

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# Effect of vitamin D on health and disease: evidence from Mendelian randomization

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## OBJECTIVES

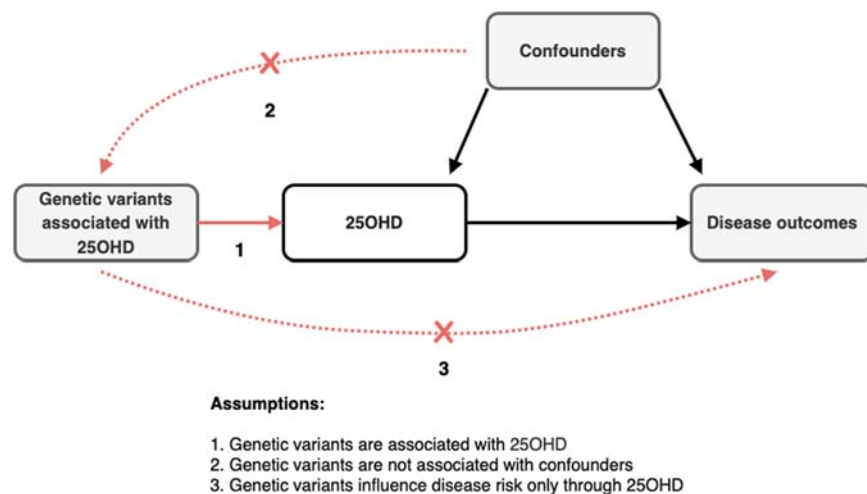
- Recognize the current knowledge gaps in the causal relationships between 25(OH)D levels and health outcomes in humans.
- Understand the basics of Mendelian randomization as a tool for causal inference.
- Present and discuss the causal evidence from Mendelian randomization studies for 25(OH)D.
- Discuss the implications of findings of Mendelian randomization studies in recommending vitamin D supplementation.

## 1. Introduction

Mendelian randomization (MR) is an established genetic epidemiological method able to test whether genetically determined levels of risk factors, such as serum 25-hydroxyvitamin D (25(OH)D) levels, are associated with disease risk [1]. There are several principles that have prompted the widespread application of MR to access causality in observational research. First, genetic variants are randomly assigned at conception in a

process similar to the randomization used in a randomized controlled trial (RCT). As such, MR limits bias due to confounding factors. This is particularly useful in the case of 25(OH)D, since it is associated with many factors that may confound its relationship with disease, such as educational attainment, body mass index (BMI), and smoking. Second, alleles are inherited at conception, which always precedes disease onset. Therefore, MR is free of bias due to reverse causation, a phenomenon where disease can influence 25(OH)D levels. This is particularly relevant since many chronic diseases can lead individuals to stay indoors more often, being less exposed to sun and having lowered 25(OH)D levels. Third, MR studies provide an assessment of a lifetime exposure to lowered 25(OH)D levels since alleles are inherited at conception.

Despite these advantages, MR studies also have important limitations, which arise mostly from the assumptions inherent to the MR design (Fig. 61.1). Given these limitations we have recently published a guideline providing readers with criteria useful for evaluating and conducting MR studies [2,3]. The first MR assumption is that the genetic variants are strongly associated with the exposure, in this case 25(OH)D levels. This can be verified by utilizing only genetic variants that have been associated at a genome-wide significant level ( $P < 5 \times 10^{-8}$ ) with 25(OH)D (see Chapter 60). The



**FIGURE 61.1** Direct acyclic graph showing illustrating the vitamin D Mendelian randomization studies and their assumptions.

second assumption is that the genetic variants are not associated with factors that confound the relationship between the exposure and the outcome. This assumption holds, as shown by the lack of association between variants in four key genes (*DHCR7*, *CYP2R1*, *GC*, and *CYP24A1*, all genes with established biological mechanisms influencing 25(OH)D levels) with potential confounders [4]. The third assumption of no (horizontal) pleiotropic relations is most challenging and failing to account for it can introduce a major bias. To properly assess the effect of genetically determined 25(OH)D on disease outcomes, the alleles that influence 25(OH)D levels must not influence risk of disease independently of 25(OH)D levels [1]. While other assumptions are required and some statistical extensions of the MR methods allow for their relaxation, it is important to bear in mind these assumptions, as they directly affect the validity of MR studies. An additional limitation of the traditional MR design is its inability to assess nonlinear effects of 25(OH)D on health outcomes, i.e., effects of low 25(OH)D levels below a threshold. In recent years, nonlinear MR studies have evolved allowing to explore causal effects of 25(OH)D levels across different cutoffs. Studies using thresholds below 50 nmol/L (defining vitamin D insufficiency) or below 25 nmol/L (defining vitamin D deficiency) have yielded contradictory results to those of traditional MR.

In this chapter, we review and discuss the evidence from the largest MR studies for 25(OH)D over the past 10 years. Despite a wealth of data supporting a role of vitamin D in human diseases in observational studies [5], evidence from large randomized control trials (RCTs) is scarce [6], with only a few exceptions [7–10]. Recent major RCTs recruiting participants with a generally better health profile, higher dosages of vitamin D supplementation, and more vitamin D replete subjects at baseline have generated mostly null results in the intention-to-treat primary outcome analysis [7–11].

Nevertheless, for many outcomes, evidence from RCT still does not exist, or the available evidence comes from small RCTs with short follow-up. Since MR can help to provide insights of the causal role of vitamin D in the etiology of common diseases, such studies can fill this knowledge gap, especially for diseases that are rare, appear later in life, and are difficult to study in RCTs.

Over 100 MR studies have evaluated the role of a life-long genetically determined 25(OH)D level on a variety of common diseases and traits [6]. To do this, MR studies have used genetic variants (single nucleotide polymorphisms [SNPs]) associated with 25(OH)D levels in genome-wide association studies (GWASs) as instruments to infer 25(OH)D serum levels. Depending on their number, these SNPs explain between 2% and 10% of the variance in 25(OH)D serum levels. Here, we are reporting results of the largest and most powered 25(OH)D MR studies for each outcome, published over the past 10 years (Table 61.1). Most of these studies are based on a two-sample MR design, where effects of the 25(OH)D SNPs on the studied outcomes are derived from cohorts that are different from the cohort from which the effects of the SNPs on 25(OH)D are generated. The two-sample MR design usually provides better statistical power compared with the one-sample MR design, in which estimates of the SNPs on both the exposure and an outcome are calculated in the same cohort. Also, the majority of the MR effects that are reported in this chapter, if not otherwise specified, correspond to the results of the inverse variance weighted (IVW) MR analysis, which is a statistically powerful MR method, albeit more susceptible to horizontal pleiotropy than approaches such as MR-Egger [93]. In most studies, results of the IVW MR method were confirmed by other MR methods (such as the MR-Egger [94], weighted median [95], and weighted mode [96]), which all aim to reduce potential horizontal pleiotropy.

**TABLE 61.1** List of Mendelian randomization studies for vitamin D, ordered by date of publication (PMID).

Study	Genetic instruments	No./No. of events	Outcomes	Estimate of effect(95% CI)	P Value	Unit of estimated effect
Ye et al. [12]	<i>CYP2R1, DHCR7, DBP, and CYP24A1</i>	104,488/28,144	T2D	1.01 (0.75, 1.36)	0.94	1-SD decrease in 25(OH)D level
		46,368	Fasting glucose	−0.02 (−0.04, 0.01)	0.28	mmol/L per SD decrease in 25(OH)D level
		46,368	2-h Glucose	0.08 (−0.06, 0.22)	0.25	mmol/L per SD decrease in 25(OH)D level
		46,368	Fasting insulin	−1.04 (−3.91, 1.83)	0.48	% difference per SD decrease in 25(OH)D level
		46,368	HbA1c	0.01 (−0.04, 0.05)	0.80	% difference per SD decrease in 25(OH)D level
Vimaleswaran et al. [13]	<i>CYP2R1 and DHCR7</i>	146,581	SBP	−0.37 (−0.73, 0.003)	0.05	mm Hg per 10% increase in 25(OH)D level
		142,255	DBP	−0.29 (−0.52, −0.07)	0.01	mm Hg per 10% increase in 25(OH)D level
		142,255	Risk of hypertension	0.92 (0.87, 0.97)	0.00	per 10% increase in 25(OH)D level
Manousaki et al. [14]	<i>DHCR7, CYP2R1, GC, and CYP24A1</i>	86,995/22,233	Coronary artery disease	0.99 (0.84, 1.17)	0.93	1-SD decrease in log-transformed 25(OH)D level
Morkry et al. [15]	<i>DHCR7, CYP2R1, GC, and CYP24A1</i>	38,589/14,498	Multiple sclerosis	2.02 (1.65, 2.46)	0.00	1-SD decrease in log-transformed 25(OH)D level
Morkry et al. [16]	<i>DHCR7, CYP2R1, GC, and CYP24A1</i>	54,162/17,008	Alzheimer's disease	1.25 (1.03, 1.51)	0.02	1-SD decrease in log-transformed 25(OH)D level
Afzal et al. [17]	<i>DHCR7 and CYP2R1</i>	95,766/10,349	All-cause mortality	1.3 (1.05, 1.61)	NA	20 nmol/L lower 25(OH)D
		95 23/97	Cardiovascular mortality	0.77 (0.55, 1.08)	NA	
		95 17/63	Cancer mortality	1.43 (1.02, 1.99)	NA	
		958/27	Other mortality	1.44 (1.01, 2.04)	NA	
Ong et al. [18]	<i>DHCR7, CYP2R1 and GC</i>	31,719/10,065	All ovarian cancer	1.27 (1.06, 1.51)	NA	20 nmol/L lower 25(OH)D
Li et al. [19]	<i>DHCR7, CYP2R1, GC, and CYP24A1</i>	1824	Lumbar 1–4 BMD	−0.048 (−0.158, 0.062)	0.38	g/cm <sup>2</sup> per unit increase in log-transformed 25(OH)D
		1824	Femoral neck BMD	−0.044 (−0.120, 0.032)	0.26	g/cm <sup>2</sup> per unit increase in log-transformed 25(OH)D
		1824	Total hip BMD	−0.041 (−0.123, 0.041)	0.33	g/cm <sup>2</sup> per unit increase in log-transformed 25(OH)D
		1824	PTH	0.088 (−0.034, 0.210)	0.15	pg/mL per unit increase in log-transformed 25(OH)D
		1824	P1NP	−0.099 (−0.291, 0.093)	0.31	g/L per unit increase in log-transformed 25(OH)D

Continued

**TABLE 61.1** List of Mendelian randomization studies for vitamin D, ordered by date of publication (PMID).—cont'd

Study	Genetic instruments	No./No. of events	Outcomes	Estimate of effect(95% CI)	P Value	Unit of estimated effect
Hysinger et al. [20]	CYP2R1 and GC	5080/1203	Pediatric asthma	−0.0000351 (NA, NA)	0.85	NA
		NA	Severe asthma exacerbations	−0.00833 (NA, NA)	0.86	NA
Dudding et al. [21]	GC, CYP2R1, DHCR7, CYP24A1, PDE3B	11,117/5133	Oral and oropharyngeal cancer	1.01 (0.74, 1.40)	0.93	Per 1-SD increase in log transformed 25(OH)D
Chandler et al. [22]	DHCR7, CYP2R1, GC	3985/23,294 women	Incident total cancer	1.10 (0.96, 1.25)	0.17	20 nmol/L higher 25(OH)D level as determined by genetic variants
		1560	Incident breast cancer	1.14 (0.92, 1.41)	0.22	
		329	Incident colorectal cancer	1.54 (0.96, 2.46)	0.07	
		330	Incident lung cancer	0.96 (0.55, 1.68)	0.89	
		770	Total cancer death	0.98 (0.73, 1.32)	0.90	
Chen et al. [23]	DHCR7, CYP2R1, GC, CYP24A1	4254 men	Total testosterone	0.12 (0.02, 0.22)	NA	1-SD increment of 25(OH)D determined by genetic variants
Larsson et al. [24]	DHCR7, CYP2R1, GC, CYP24A1	32,965	Femoral neck BMD	0.02 (−0.03, 0.07)	0.37	1-SD increment of 25(OH)D determined by genetic variants
		32,965	Lumbar spine BMD	0.02 (−0.04, 0.08)	0.49	
		142,487	Estimated BMD	−0.03 (−0.05, −0.01)	0.02	
Sun et al. [25]	DHCR7, CYP2R1, GC, CYP24A1, SEC23A, AMDHD1	66,628	Total body BMD	0.92 (0.82, 1.04)	0.17	NA
Dimitrakopoulou et al. [26]	DHCR7, CYP2R1, GC, CYP24A1	11,488	Colorectal cancer	0.92 (0.76, 1.10)	0.36	Per 25 nmol/L increase in genetically determined 25(OH)D level
		15,748	Breast cancer	1.05 (0.89, 1.24)	0.59	
		22,898	Prostate cancer	0.89 (0.77, 1.02)	0.08	
		4369	Ovarian cancer	1.12 (0.86, 1.47)	0.40	
		12,537	Lung cancer	1.03 (0.87, 1.23)	0.72	
		1896	Pancreatic cancer	1.36 (0.81, 2.27)	0.25	
		1627	Neuroblastoma cancer	0.76 (0.47, 1.21)	0.24	
Ong et al. [27]	GC, CYP2R1, DHCR7, and CYP24A1	438,870/46,155	Cancer	0.97 (0.91, 1.04)	0.40	Per 20 nmol/L increase in 25(OH)D
		438,870/6998	Cancer mortality	0.97 (0.84, 1.11)	0.54	
Aspelund et al. [28]	DHCR7, CYP2R1	10,501/4003	All-cause mortality	1.32 (0.80, 2.24)	NA	Per 20 nmol/L decrease in genetically determined 25(OH)D level
		10,501/4003	All-cause mortality	1.35 (0.81, 2.37)	NA	NA



He et al. [29]	DHCR7, CYP2R1, GC, CYP24A1, SEC23A, AMDHD1	48,168/18,967	Colorectal cancer	0.91 (0.69, 1.19)	0.48	Per unit log-transformed 25(OH)D change determined by genetic variants
Cuellar-Partida et al. [30]	DHCR7, CYP2R1, GC, CYP24A1	37,382 European	Myopic refractive error	−0.02 (−0.09, 0.04)	NA	Per 10 nmol/L increase in 25(OH)D level
		8376 Asian	Myopic refractive error	0.01 (−0.17, 0.19)	NA	
Winslow et al. [31]	DHCR7, CYP2R1	1569/103,084	Nonmelanoma skin cancer	1.11 (0.91, 1.35)	NA	Per 20 nmol/L higher 25(OH)D level as determined by genetic variants
Havdahl et al. [32]	DHCR7, CYP2R1, GC, CYP24A1, SEC23A, AMDHD1	19,526/327,478	Fatigue	1.05 (0.87, 1.27)	0.62	1-SD decrease log transformed 25(OH)D level
Takahashi et al. [33]	DHCR7, CYP2R1, GC, CYP24A1	12,488/18,169	Glioma	1.21 (0.90, 1.62)	0.20	NA
Larsson et al. [34]	DHCR7, CYP2R1, GC, CYP24A1	17,008/37,154	Alzheimer's disease	0.92 (0.85, 0.98)	0.01	Per 20% higher levels
Teumer et al. [35]	DHCR7, CYP2R1, GC	133,720	Estimated glomerular filtration rate	−0.013	0.00	NA
		54,448	Urinary albumin: creatinine ratio	0.032	0.27	
Manousaki et al. [36]	GC, CYP2R1, DHCR7, and CYP24A1	146,761/25,109	Asthma	1.03 (0.90, 1.19)	0.63	1-SD decrease in log-transformed 25(OH)D level
		15,008/7047	Childhood onset asthma	0.95 (0.69, 1.31)	0.76	
		40,835/10,788	Atopic dermatitis	1.12 (0.92, 1.37)	0.27	
		12,853	Elevated IgE level	−0.40 (−1.65, 0.85)	0.54	
Bae et al. [37]	SSTR4, GC, and NADSYN1	4744/2104	Systemic lupus erythematosus	0.032 (−0.201, 0.265)	0.79	NA
		41,282/12,307	Rheumatoid arthritis	0.026 (−0.094, 0.146)	0.66	
Magnus et al. [38]	GC, CYP2R1, DHCR7, and CYP24A1	9447	Gestational hypertension	0.90 (0.78, 1.03)	NA	10% decrease in 25(OH)D
		9447	Preeclampsia	0.98 (0.89, 1.07)	NA	
Lund-Nielsen et al. [39]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	115 16/95	Crohn's disease	0.98 (0.94, 1.03)	NA	1.4-nmol/L increase in 25(OH)D
		115,110/1265	Ulcerative colitis	1.01 (0.97, 1.05)	NA	
Larsson et al. [40]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	54,162/17,008	Alzheimer's disease	0.86 (0.78, 0.94)	0.00	1-SD increase in 25(OH)D level

Continued

**TABLE 61.1** List of Mendelian randomization studies for vitamin D, ordered by date of publication (PMID).—cont'd

Study	Genetic instruments	No./No. of events	Outcomes	Estimate of effect(95% CI)	P Value	Unit of estimated effect
Michaelsson et al. [41]	GC, CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	173,005/59,851	Major depression	1.02 (0.97, 1.08)	0.44	1-SD decrease in 25(OH)D level
Larsson et al. [42]	GC, CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	438,847/34,217	Ischemic stroke	1.01 (0.94, 1.08)	0.84	1-SD increase in 25(OH)D level
Tan et al. [43]	GC, CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	72,729	Circulating insulin-like growth factor-binding protein 3 (IGFBP-3)	0.11 (−0.10, 0.31)	0.32	1-unit increase in log-transformed 25(OH)D level
Larsson et al. [34]	GC, CYP2R1, DHCR7, and CYP24A1	17,352/5333	Parkinson's disease	0.98 (0.93, 1.04)	0.56	10% decrease in 25(OH)D level
Noordam et al. [44]	GC, NADSYN1, and CYP2R1	4492	Perceived age	0.030 (−0.015, 0.075)	0.18	1-unit increase in genetic risk score
		4492	Degree of skin wrinkling	0.000 (−0.054, 0.054)	1.00	
		4492	Degree of pigmented spots	0.055 (−0.004, 0.114)	0.07	
Dong et al. [45]	GC, CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	23,326/6167	Esophageal adenocarcinoma	0.68 (0.39, 1.19)	0.18	20-nmol/L increase in 25(OH)D
		23,326/4112	Barrett's esophagus	1.21 (0.77, 1.92)	0.41	
Lu et al. [46]	DHCR7, CYP2R1	58,312/370,592	Diabetes	0.86 (0.77, 0.97)	0.01	25-nmol/L high 25(OH)D as determined by genetic variants
	DHCR7, CYP2R1, GC, CYP24A1	32,796/248,629	Diabetes	0.92 (0.84, 1.01)	0.07	
Trajanoska et al. [47]	DHCR7, CYP2R1, GC, CYP24A1	562,258/185,057	Fracture risk	0.84 (0.70, 1.02)	0.07	Per SD decrease of genetically determined 25(OH)D level
Jacobs et al. [48]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	41,505/14,802	Multiple sclerosis	0.57 (0.41–0.81)	0.00	1-unit increase in the natural log-transformed vitamin D level
Mazidi et al. [49]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	37,684	Telomere length (TL)	−0.104 (−0.2706, 0.0626)	0.22	1-unit increase in the natural log-transformed vitamin D level
Kämpe et al. [50]	GC and CYP2R1 (haplotype analyses)	648 children	pQCT BMD		0.08	Haplotype combination analysis*
			pQCT BMC		0.79	
			pQCT cross-sectional area		0.03	
			pQCT cortical density		0.01	
			pQCT cortical content		0.05	

Wang et al. [51]	GC, DHCR7, CYP2R1, CYP24A1, AMDHD1, and SEC23A	IGAP 41 944/21,982 314,278/UKBB 27 696 maternal cases 14,338 paternal cases	Alzheimer's disease	0.62 (0.46–0.84)	0.19	1-SD increase of genetically determined 25(OH)D level
			Alzheimer's disease	0.88 (0.73–1.06)		
Kwak et al. [52]	DHCR7, CYP2R1, GC, CYP24A1	2591 Korean adults	SBP	−0.42 (−1.51, 0.67)	0.45	Unit change in polygenic risk score *
			DBP	0.001 (−0.67, 0.67)	0.99	
			Hypertension	1.04 (0.91, 1.19)	0.60	
Larsson et al. [53]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	36,052/12,577	Amyotrophic lateral sclerosis	0.96 (0.86–1.08)	0.52	1-SD increase in standardized 25(OH)D level
Chen et al. [54]	DHCR7, CYP2R1, GC, CYP24A1	10,655 Chinese adults	Metabolic syndrome	0.977 (0.966, 1.030)		Per 10 nmol/L GRS synthesis determined increase of 25(OH)D levels
			Waist circumference	0.403 (−0.854, 1.659)		
			Fasting plasma glucose	−0.186 (−0.399, 0.026)		
			ln(triglycerides)	−0.026 (−0.099, 0.047)		
			High-density lipoprotein	−0.010 (−0.053, 0.033)		
			Systolic blood pressure	−0.198 (−3.032, 2.637)		
Yuan et al. [55]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	898,130/74,124	Diastolic blood pressure	0.061 (−1.776, 1.899)	0.03	1-SD increment of serum 25(OH)D levels
			T2D	0.94 (0.88, 0.99)		
Meng et al. [56]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	319,778	SBP	−0.648 (−1.5320, 0.0736)	0.210	1-SD increase of the log-transformed 25(OH)D level
		319,779	DBP	−0.117 (−0.3580, 0.1240)	0.66	
		339,256/106,405	Hypertension	0.973 (0.911, 1.040)	0.340	
		339,256/15,958	T2D	0.971 (0.845, 1.117)	0.617	
		339,256/28,337	Ischemic heart disease	1.020 (0.917, 1.135)	0.647	
		338,172	BMI	0.130 (−0.1072, 0.3672)	0.329	
		339,256/23,294	Depression	0.913 (0.816, 1.022)	0.093	
		339,256/23,603	Nonvertebral fracture	0.969 (0.867, 1.083)	0.495	
		339,256/9830	All-cause mortality	1.030 (0.869, 1.222)	0.671	

Continued

**TABLE 61.1** List of Mendelian randomization studies for vitamin D, ordered by date of publication (PMID).—cont'd

Study	Genetic instruments	No./No. of events	Outcomes	Estimate of effect(95% CI)	P Value	Unit of estimated effect
Milaneschi et al. [57]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	480,359/135,458	Major depression	0.03 (−0.068, 0.128)	0.50	1-unit increase in the natural log-transformed vitamin D level
Huang et al. [58]	CYP2R1 and DHCR7	99,012 Chinese adults	Cardiovascular disease	1.01 (0.99, 1.02)	0.18	25-nmol/L increase in 25(OH)D concentrations
		106,911 Danish adults	Myocardial infraction	1.00 (0.97, 1.03)	0.54	
			Stroke	1.00 (0.99, 1.01)	0.68	
			Ischemic stroke	1.00 (0.98, 1.02)	0.69	
			Intracerebral hemorrhage	1.01 (0.98, 1.04)	0.86	
			Ischemic heart disease	1.00 (0.98–1.01)	0.54	
Liyanage et al. [59]	DHCR7, CYP2R1, GC, and CYP24A1	36,077/12,874	Melanoma	1.06 (0.95–1.19)		20-nmol L <sup>−1</sup> decrease in 25(OH)D
Thompson et al. [60]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	122,123	First childbirth weight	−0.03gr (−2.48, 2.42)	0.10	Per 10% higher maternal 25(OH)D
Libuda et al. [61]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	322,580/161,460	Depressive symptoms	1.03 (0.95, 1.11)	0.52	1-unit increase in the natural log-transformed vitamin D level
		322,580/113,769	Broad depression	1.02 (1.01, 1.04)	0.10	
Wang et al. [62]	DHCR7, CYP2R1, GC, and CYP24A1	14,570/3915 Chinese	Prediabetes	0.982 (0.948, 1.016)		1-unit increase in the natural log-transformed vitamin D level
		12,220/1565 Chinese	T2D	0.985 (0.940, 1.032)		
		16,135 Chinese	Fasting plasma glucose	−0.015 (−0.035, 0.006)		
		16,135 Chinese	hbA1c	−0.003 (−0.017, 0.011)		
Jiang et al. [63]	CYP2R1, DHCR7, CYP24A1, SEC23A, and AMDHD1	122,977	Breast cancer	1.02 (0.97–1.08)	0.47	Per 25 nmol/L increase in 25(OH)D
		79,148	Prostate cancer	1.00 (0.93–1.07)	0.99	
Mulugeta et al. [64]	GC, DHCR7, CYP2R1, CYP24A1, AMDHD1, and SEC23A	424,967/139,424	Depression	0.95 (0.94, 0.96)	NA	Per 50% higher genetically determined serum 25(OH)D
Palaniswamy et al. [65]	62 SNPs	204,402	C-reactive protein (hs-CRP)	−0.001 (−0.007, 0.005)	0.686	1-SD increase in 25(OH)D
		24,925	α1-acid glycoprotein (AGP)	−0.001 (−0.008, 0.006)	0.748	
		5163	Soluble intercellular adhesion molecule 1 (sICAM-1)	−0.002 (−0.009, 0.005)	0.585	

Zheng et al. [66]	GC, CYP2R1, NADSYN1/DHCR7, AMDHD1, SEC23A, CYP24A1, PADI1, CRCT1, UGT1A5, and SULT2A1	80,983/842,909	Type 2 diabetes	0.96 (0.89, 1.03)	0.23	1-SD higher level of 25(OH)D
Xiao et al. [67]	CYP2R1 and DHCR7	361/2393 Chinese	Type 2 diabetes	1.10 (1.02, 1.45)	0.01	Per 25-nmol/L decrease in 25(OH)D concentration
	CYP2R1 and DHCR7	2393/746 Chinese	Metabolic syndrome	0.85 (0.56, 1.28)	0.59	
	CYP2R1 and DHCR7	2393 Chinese	Diastolic blood pressure	1.14 (1.03, 1.43)	0.02	
	CYP2R1 and DHCR7	2393 Chinese	Systolic blood pressure	0.76 (0.51, 1.31)	0.14	
	CYP2R1, DHCR7, GC, and CYP24A1	361/2393 Chinese	Type 2 diabetes	0.91 (0.60, 1.36)	0.74	
	CYP2R1, DHCR7, GC, and CYP24A1	2393/746 Chinese	Metabolic syndrome	0.92 (0.60, 1.22)	0.16	
	CYP2R1, DHCR7, GC, and CYP24A1	2393 Chinese	Diastolic blood pressure	0.83 (0.62, 1.10)	0.58	
	CYP2R1, DHCR7, GC, and CYP24A1	2393 Chinese	Systolic blood pressure	1.04 (0.70, 1.45)	0.37	
He et al. [68]	110 SNPs	67,878/26,393	Colorectal cancer	0.97 (0.88–1.07)	0.57	Per unit of rank-based inverse-normal transformed vitamin D concentration
Ong et al. [69]	74 SNPs	140,254	Prostate cancer	1.07 (0.89–1.29)	0.46	1-SD change in 25(OH)D
		228,951/122,977	Breast cancer	1.03 (0.93–1.13)	0.60	
		27,209/11,348	Lung cancer	0.94 (0.78–1.13)	0.50	
		66,450/25,509	Epithelial ovarian cancer	0.74 (0.42–1.29)	0.03	
		3835/1896	Pancreatic cancer	0.93 (0.46–1.92)	0.99	
		4881/1627	Neuroblastoma cancer	0.74 (0.42–1.29)	0.29	
		3305/12,501	Juvenile idiopathic arthritis	1.00 (0.76, 1.33)	0.94	
Clarke et al. [70]	69 SNPs	3305/12,501	Juvenile idiopathic arthritis	1.00 (0.76, 1.33)	0.94	1-SD increase in standardized natural log-transformed 25(OH)D levels
Jiang et al. [71]	GC, CYP2R1, DHCR7, and AMDHD1	10,619/15,145	Ankylosing spondylitis	0.999 (0.997, 1.002)	0.72	1-SD increase in standardized natural log-transformed 25(OH)D levels
Bergink et al. [72]	GC, CYP2R1, DHCR7, and AMDHD1	>562,000/23,877	Knee osteoarthritis	1.03 (0.84, 1.26)	0.75	1-SD decrease in log-transformed 25(OH)D level
		>562,000/17,517	Hip osteoarthritis	1.06 (0.83, 1.35)	0.63	

Continued



**TABLE 61.1** List of Mendelian randomization studies for vitamin D, ordered by date of publication (PMID).—cont'd

Study	Genetic instruments	No./No. of events	Outcomes	Estimate of effect(95% CI)	P Value	Unit of estimated effect
Feng et al. [73]	SEC23A, GC, CYP4A1, NADSYN1/DHCR7, CYP2R1, AMDHD1, and CYP2R1	297,526/29,856	Allergic rhinitis	0.96 (0.78, 1.18)	0.70	NA
		8040/24,481	Allergic sensitization	1.06 (0.69, 1.63)	0.80	
		2028/11,634	Nonallergic rhinitis	0.94 (0.59, 1.49)	0.78	
Manousaki et al. [74]	69 SNPs	9358/24,063	Type 1 diabetes	1.09 (0.86, 1.40)	0.48	1-SD decrease in standardized natural log-transformed 25(OH)D
Zhong et al. [75]	NA	999/5416 Chinese	Behçet's disease	3.82 (1.27, 11.42)	NA	1-SD increase of natural log-transformed 25(OH)D level
		1215/2493 Turkish		4.18 (1.15, 15.12).	NA	
		2214/7907 combined		3.96 (1.72, 9.13)	0.00	
Zhang et al. [76]	GC	NA	Alzheimer's disease	0.63 (0.45, 0.89)	0.01	1-SD increase 50 mg/L
Dodhia et al. [77]	79 SNPs	17,035	Caries in primary teeth	1.06 (0.81, 1.31)	0.66	1-SD increase in natural log-transformed 25(OH)D
		13,386	Caries in permanent teeth in childhood and adolescence	1.00 (0.76, 1.23)	0.97	
		26,792	Caries severity in adulthood	0.31 fewer affected tooth surfaces (1.81, 1.19)	0.68	
Pilling et al. [78]	GC, DHCR7, CYP2R1, CYP24A1, AMDHD1, and SEC23A	3634/351,320	Incident delirium	0.80 (0.73, 0.87)	$2 \times 10^{-7}$	Per unit of natural log-transformed 25(OH)D levels
Libuda et al. [79]	GC, DHCR7, CYP2R1, CYP24A1, AMDHD1, and SEC23A	53,293/19,099	Attention-deficit/hyperactivity disorder (ADHD)	-0.043 (-0.353, 0.439)	0.83	Per unit of natural log-transformed 25(OH)D levels
Arathimos et al. [80]	110 SNPs	2120/126,136	Atypical depression	1.04 (0.80, 1.36)	0.76	Per unit of rank-based inverse-normal transformed vitamin D concentration
		1891/178,585	Treatment-resistant and atypical depression	1.01 (0.78, 1.31)	0.95	
Baumeister et al. [81]	288 SNPs	45,563/17,353	Periodontitis	1.04 (0.97, 1.12)	0.30	1-SD increase in natural log-transformed 25(OH)D
Butler-Laporte et al. [82]	80 SNPs	1,299,010/14,134	COVID-19 susceptibility	0.95 (0.84, 1.08)	0.44	1-SD increase in natural log-transformed 25(OH)D levels
		908,494/6406	COVID-19 hospitalization	1.09 (0.89, 1.33)	0.41	
		628,238/4336	COVID-19 severe disease	0.97 (0.77, 1.22)	0.77	

Amin et al. [83]	17 SNPs	127,637/11,181	COVID-19 infection	−0.04 (−0.10, 0.03)	0.25	NA
		7268/1389	COVID-19 severity	−0.24 (−0.55, 0.08)	0.14	
Chan et al. [84]	12 SNPs	441/107 Chinese	Recurrent or de novo ischemic stroke or MI	0.64 (0.42–0.91)	NA	NA
Jian et al. [85]	10 SNPs	337,199	Calcium levels	0.014 (0.010–0.018)	7.64E-10	1-SD change in 25(OH)D level
		395,044/6536	Kidney stone disease	1.47 (1.22, 1.77)	5.49E-05	
Hu et al. [86]	11 SNPs	27,949	Number of teeth	0.085 (0.019, 0.150)	0.01	Per 1-SD increase in genetically predicted serum vitamin D levels
		26,792 (DFMS) 26,533 (DFSS)	Dental caries	−0.057 (−0.119, 0.005 for DFMS) −0.017 (−0.077, 0.043 for DFSS)	0.07 (DMFS) 0.58 (DFSS)	
Yuan et al. [87]	GC, DHCR7, CYP2R1, CYP24A1, AMDHD1, and SEC23A	45,563/17,353	Periodontitis	0.031 (−0.071, 0.132)	0.55	1-SD increase in genetically predicted S-25(OH)D levels
		19,264/1483	Fatty liver disease	0.78 (0.69, 0.89)	<0.001	
		344,292	Alanine aminotransferase (ALT)	−0.03 (−0.16, 0.11)	0.71	
Wang et al. [88]	20 SNPs	344,136	Aspartate aminotransferase (AST)	−0.17 (−0.36, 0.01)	0.07	NA
		38,589/14,498	Multiple sclerosis	0.46 (0.33, 0.63)	0.00	
Kirwan et al. [89]	GC, DHCR7, CYP2R1, CYP24A1, AMDHD1, and SEC23A	NA	Total fat-free mass	0.042 (0.005, 0.079)	0.04	25-nmol/L increase in serum 25(OH)D
			Trunk fat-free mass	0.045 (0.006, 0.084)	0.02	
			Left arm fat-free mass	0.050 (0.015, 0.085)	0.01	
			Right arm fat-free mass	0.044 (0.015, 0.073)	0.00	
			Left leg fat-free mass	0.039 (−0.006, 0.084)	0.10	
			Right leg fat-free mass	0.030 (−0.021, 0.081)	0.24	
Revez et al. [90]	236	NA	ADHD	0.01 (−0.08–0.10)	0.04	Per unit increase in rank-based inverse-normal transformed 25(OH)D level; without BMI adjustment
	268		Allergic rhinitis	0.04 (−0.02–0.09)	0.03	
	270		Alzheimer's disease	−0.02 (−0.04–0.01)	0.01	
	236		Autism spectrum disorder	0.01 (−0.08–0.10)	0.04	
	266		Bipolar disorder	−0.03 (−0.11–0.06)	0.04	
	258		Coffee intake	0.00 (−0.01–0.02)	0.01	
	232		Coronary artery disease	−0.02 (−0.06–0.02)	0.02	
	220		Dyslipidemia	−0.06 (−0.09–0.02)	0.02	

Continued

**TABLE 61.1** List of Mendelian randomization studies for vitamin D, ordered by date of publication (PMID).—cont'd

Study	Genetic instruments	No./No. of events	Outcomes	Estimate of effect(95% CI)	P Value	Unit of estimated effect
Ye et al. [91]	143 SNPs	NA	230 Educational attainment	−0.01 (−0.02–0)	0.01	NA
			239 Fluid intelligence	−0.01 (−0.02–0.01)	0.01	
			246 Hypertension	−0.03 (−0.06–0)	0.02	
			270 Inflammatory bowel disease	−0.08 (−0.19–0.02)	0.05	
			277 Macular degeneration	−0.03 (−0.17–0.1)	0.07	
			240 Major depressive disorder	−0.02 (−0.05–0)	0.01	
			268 Parkinson's disease	0.12 (0.01–0.24)	0.06	
			218 Rheumatoid arthritis	−0.01 (−0.24–0.22)	0.12	
			245 Schizophrenia	−0.05 (−0.11–0.01)	0.03	
			155 Type II diabetes	−0.04 (−0.09–0.02)	0.03	
			Height	0.064 (0.019, 0.11)	NA	
			Ovarian cancer	0.96 (0.93, 0.99)		
			Multiple sclerosis	0.96 (0.94, 0.98)		
			Leg fracture	0.60 (0.45, 0.80)		
			Femur fracture	0.53 (0.32, 0.80)		
Zhuang et al. [92]	DHCR7, CYP2R1, GC/DBP, CYP24A1	NA	Cholesterol	NA	NA	225 tested lipid and other metabolites. Only few selected outcomes are shown
			Lipoprotein particle			
			Phospholipids within very small VLDL and IDL			

The concordance between MR studies of 25(OH)D levels and RCTs of vitamin D supplementation with multiple outcomes is striking, while diverging from the findings of observational studies. This supports the contention that MR studies are better suited than observational studies to understand the effect of 25(OH)D levels on risk of disease. Nevertheless, most RCTs and MR studies have been undertaken in individuals from the general population in which the prevalence of severe vitamin D deficiency is low. Most available MR studies have mostly reported null results, but as mentioned before, their design is handicapped by their inability to study nonlinear effects of 25(OH)D levels. However, combining evidence arising from RCTs and MR studies directly informs on the anticipated effects of vitamin D supplementation in the general population.

## 2. Type 2 diabetes

Since 2015, at least five well-powered MR studies have investigated the causal role of genetically determined 25(OH)D levels on risk of type 2 diabetes (T2D) and related traits. The earliest MR study [12], comprising several European populations of European descent, utilized four SNPs within or near genes related to 25(OH)D synthesis and metabolism (*DHCR7*-rs12785878, related to vitamin D synthesis; *CYP2R1*-rs10741657, the hepatic 25-hydroxylase; *GC*-rs2282679 involved in 25(OH)D transport; and *CYP24A1*-rs6013897 involved in catabolism) to infer 25(OH)D levels. These SNPs together explain 2.4% of the variance in 25(OH)D levels. Each SNP was assessed for an association with risk of T2D in a sample of 28,144 cases and 76,344 controls, and with glycemic traits (concentrations of fasting glucose, 2-h glucose at OGTT, fasting insulin, and HbA<sub>1c</sub>) among 46,368 individuals. These associations were then combined in an MR analysis to estimate the causal association of 25(OH)D concentration with T2D risk and with the glycemic traits. The MR effect (OR) for T2D was 1.01 (95% CI 0.75–1.36) per 25 nmol/L (equivalent to 1 standard deviation [SD]) lower 25(OH)D concentration. The MR-derived estimates for all four glycemic traits were not significant (all  $P > .25$ ). Therefore, it was concluded that association between genetically determined 25(OH)D concentration and T2D is unlikely to be causal.

Another MR study [46] investigated SNPs in the same four vitamin D genes in a metaanalysis of 10 studies (combining European and Chinese populations). The per allele effect across genetic scores on 25(OH)D levels was 2.87 nmol/L for the two synthesis SNPs (in *DHCR7* and *CYP2R1*) examined in 58,312 cases and 3.54 nmol/L for a four SNP score examined in 32,796 cases. Studying a nonlinear effect of genetically determined levels with a

cutoff of 25 nmol/L using the synthesis SNPs, indicated that a 25(OH)D concentration above this threshold was protective with an MR OR of 0.86 (95% CI 0.77–0.97) for T2D. An equivalent difference in 25(OH)D using a genetic score constructed from the four SNPs was not significantly associated with T2D (MR OR 0.92, 95% CI 0.84–1.01). These discordant results are difficult to interpret, but the absence of causal association based on the four SNP scores is notable, given that these SNPs explain a larger portion of the variance in 25(OH)D levels than the two synthesis SNPs alone.

In 2019, an umbrella MR paper [56] investigated associations of 25(OH)D levels and multiple traits in 339,256 individuals of White British origin from UK Biobank, among which T2D status. In this study, six SNPs were used as instrument to infer 25(OH)D levels, including the four previous SNPs and two newly identified SNPs in *SEC23A* and *AMDHD* (both genes do not have clear role in vitamin D synthesis and metabolism). These six SNPs together explain 5.3% of the variance in 25(OH)D levels. This MR analysis, with substantial study power (>80% power to detect an association with an OR >1.2 per SD increase of log-transformed 25(OH)D), did not support a causal association with T2D (MR OR 0.97, 95% CI 0.85 to 1.12 per 1SD increase in the log-transformed 25(OH)D level).

A two-sample MR study [55] on a larger population totaling 898,130 individuals of European ancestry (74,124 T2D cases and 824,006 controls) from the DIAGRAM consortium used SNPs in the same six vitamin D genes as the previous study, and a low-frequency variant of large effect on 25(OH)D level in *CYP2R1* (rs10741657). Using an IVW method, they demonstrated, per 1 SD increment of 25(OH)D levels, a protective MR OR for T2DM of 0.94 (95% CI 0.88–0.99). Results from the weighted median method and from the MR-Egger regression (both methods are sensitive to directional pleiotropy) were not significant.

Finally, a recent one-sample MR study [90] using up to 285 SNPs, all significantly associated with 25(OH)D levels, investigated the causal role of 25(OH)D in multiple outcomes in 417,580 White British individuals from UK Biobank. These SNPs explain nearly 13% of the variance in 25(OH)D levels. Using sensitivity analyses excluding SNPs with possible pleiotropic effects, and adjusting or not for BMI, this MR analysis failed to provide evidence for an unconfounded association between genetically lowered 25(OH)D levels and T2D (MR ORs 0.96, 95% CI 0.91 to 1.02 without adjustment for BMI; 0.98, 95% CI 0.93 to 1.03 with SNPs conditioned on BMI; and 1, 95% CI 0.94 to 1.05 when using BMI as a covariate). These results were similar to those of an MR study [66] using only 10 25(OH)D SNPs and including up to 80,983 T2D cases and 842,909 noncases. This study showed that a genetically predicted 1-SD increase in

25(OH)D was not significantly associated with T2D (MR OR 0.96, 95% CI 0.89–1.03).

In non-Europeans, an MR study in 16,135 Chinese individuals [62] used four SNPs in *DHCR7*, *CYP2R1*, *GC*, and *CYP24A1* and tested effects of 25(OH)D on T2D risk and glycemic traits. The MR-derived OR for risk of T2D (in a sample of 1565 cases and 10,655 controls) and prediabetes (among 3915 cases and 10,655 controls) was 0.99 (95% CI 0.94–1.03) and 0.98 (95% CI 0.95–1.02), respectively. The MR estimates for fasting plasma glucose and HbA<sub>1c</sub> were also not significant. The most recent MR study in Chinese participants [67] studied effects on T2D risk in 2393 individuals (361 cases) and demonstrated an OR of 1.10 (95% CI 1.02–1.45) for T2D when constructed from the two synthesis SNPs in *CYP2R1* and *DHCR7*, but no effect when using four SNPs comprising both synthesis and metabolism genes (*CYP2R1*, *DHCR7*, *GC*, and *CYP24A1*).

Overall, the aforementioned evidence converges to an absence of causal association between 25(OH)D levels and T2D or glycemic traits in Europeans and Asians.

### 3. Risk of cancers

A handful of MR studies have investigated the association between serum 25(OH)D concentration and the risk of cancer in individuals of European background. For start, the Ovarian Cancer Association (OCA) Consortium (10,065 cases, 21,654 controls) [18] reported a 27% increase in the risk of epithelial ovarian cancer risk per 20 nmol/L decrease in genetically determined 25(OH)D serum concentration (MR OR 1.27, 95% CI 1.06–1.51). The effect was stronger in high-grade serous epithelial ovarian cancer (MR OR 1.54, 95% CI 1.19–2.01). However, these findings were not fully corroborated by a follow-up study [26] comprising 4369 cases and 84,418 controls, reporting a nonsignificant MR OR of 1.12 (95% CI 0.86–1.47) per 25 nmol/L increase in genetically determined 25(OH)D levels. This discrepancy can be due to differences in power or to the quality of the genetic instrument (derived from 2346 participants in the first study [18], versus up to 38,000 individuals from a large-scale metaanalysis in the latter study [26]). The latter publication [26] also showed no evidence of association between genetically determined 25(OH)D concentrations and risk of colorectal (MR OR 0.92, 95% CI 0.76 to 1.10), breast (MR OR 1.05, 95% CI 0.89–1.24), prostate (MR OR 0.89 95% CI 0.77–1.02), lung (MR OR 1.03, 95% CI 0.87–1.23), pancreatic (MR OR 1.36, 95% CI 0.81–2.27), and cancer and neuroblastoma (MR OR 0.76, 95% CI 0.47–1.21). Similar findings were reported in another study [22] using five SNPs as instrumental variables mapping to the

*DHCR7*, *CYP2R1*, and *GC* genes in relation with total incident cancer (3985 cases, MR OR 1.10, 95% CI 0.96–1.25) and cancer subtypes such as breast (1560 cases, MR OR 1.14, 95% CI 0.92–1.41), colorectal (329 cases, MR OR 1.54, 95% CI 0.96–2.46), and lung (330 cases, MR OR 0.96, 95% CI 0.55–1.68) cancer in 23,294 women. The null effect on colorectal carcinoma was confirmed in a relatively large MR study (18,967 cases and 48,168 controls) using seven SNP mapping to *DHCR7*, *CYP2R1*, *GC*, *CYP24A1*, *SEC23A*, and *AMDHD1* [29]. Similarly, a large-scale MR study (122,977 breast cancer cases and 79,148 prostate cancer cases) did not show a causal effect of 25(OH)D concentrations on breast (MR OR 1.02, 95% CI 0.97–1.08) and prostate (MR OR 1.00, 95% CI 0.93–1.07) cancer risk [63]. 25(OH)D concentrations were also not associated with the risk of oral and oropharyngeal cancer [21] (5133 cases and 5984 controls) and esophageal adenocarcinoma (4112 cases and 17,159 controls) [45]. Moreover, the OR per 20 nmol/L increase in genetically estimated 25(OH)D concentration was 1.11 (95% CI 0.91–1.35) for nonmelanoma skin cancer (6167 cases and 23,326 controls) [31] and 0.94 (95% CI 0.87–1.05) for melanoma (12,874 cases and 23,203 controls) [59], indicating that vitamin D levels may not be causally associated with the risk of skin cancer. Genetically determined 25(OH)D was also not associated with increased glioma risk (MR OR 1.21, 95% CI 0.90–1.6) [33]. All aforementioned MR observations have been reevaluated and confirmed using a larger set of variants associated with 25(OH)D concentrations (up to 110 SNPs) [26,29,68,69,97]. Overall, the current MR studies support a potential protective effect of vitamin D on ovarian cancer but not for any other types such as breast, prostate, and colorectal cancer among others.

### 4. Risk of cardiovascular events

To date, six MR studies have investigated the effect of genetically determined 25(OH)D levels on cardiovascular events and related outcomes. A study using SNPs in the two vitamin D synthesis genes (*DHCR7* and *CYP2R1*) as instruments for 25(OH)D levels investigated the cardiovascular mortality rates in a population of 95,766 Europeans [17]. The study demonstrated that a decrease by 20 nmol/L in genetically determined 25(OH)D levels does not increase deaths from cardiovascular events (MR OR 0.77, 95% CI 0.55–1.08). Another MR study [14] on 89,995 Europeans (22,244 cases), using four vitamin D SNPs in *DHCR7*, *CYP2R1*, *GC*, and *CYP24A1*, also failed to provide evidence in favor of a causal association between 25(OH)D and coronary artery disease (MR OR 0.99, 95% CI 0.84 to 1.17, per 1-SD decrease in log-transformed 25(OH)D level).



An umbrella MR study using six vitamin D SNPs [56] (the four aforementioned SNPs, and two SNPs in *SEC23A*, and *AMDHD1*), and a substantially larger sample size (339,256 White British from UK Biobank, among which 28,337 had ischemic heart disease), showed a null association between 25(OH)D levels and ischemic heart disease (MR OR 1.02, 95% CI 0.92–1.14). An MR study on risk of ischemic stroke in 438,847 Europeans from the MEGASTROKE consortium [42], using the same six 25(OH)D-related SNPs corroborated this finding (MR OR 1.01, 95% CI 0.94–1.08). Contrarily, genetically determined lower 25(OH)D serum levels have been associated with the recurrence of ischemic vascular events in persons with prior ischemic stroke or MI [84] (MR OR 0.55, 95% CI 0.35–0.81 and MR OR 0.52, 95% CI 0.30–0.81, respectively). An one-sample umbrella MR study using a substantially larger number of 25(OH)D SNPs (242<sub>total</sub> or 232<sub>bmiadjusted</sub>) [90], explaining up to 13% of the variance in 25(OH)D levels, reported similar negative results to the studies mentioned before with an MR OR for coronary artery disease of 0.98 (95% CI 0.95–1.02) in a sample of 417,580 white British participants from the UK Biobank. Finally, using a combined Chinese and Danish population, totaling 205,923 individuals, an MR study [58] equally failed to provide evidence for a causal association between 25(OH)D and six cardiovascular traits, using two vitamin D synthesis SNPs (*DHCR7* and *CYP2R1*). Specifically, they demonstrated an MR OR of 1 for cardiovascular disease, myocardial infarction, stroke, ischemic stroke, intracerebral hemorrhage, and ischemic heart disease, with a tight 95% CI. Although the definition and type of cardiovascular events may differ among the above MR studies, and with a few exceptions, the null estimates are consistent.

## 5. Hypertension

The MR evidence for a causal effect of 25(OH)D levels on hypertension and systolic and diastolic blood pressure is consistent across five large studies, and overall does not support such effect. Specifically, an MR study [13] using as instruments two SNPs in the two vitamin D synthesis genes (*CYP2R1* and *DHCR7*) and drawn in a population of 146,581 Europeans with available measurements of systolic and diastolic blood pressure, and information on hypertension diagnosis, showed a marginal decrease in diastolic heart pressure 0.29 mm Hg per 10% increase in 25(OH)D levels. There was no significant effect on systolic heart pressure, and the MR OR for hypertension was 0.92 (95% CI 0.87–0.97). More recently, using six 25(OH)D-related SNPs (in *DHCR7*, *CYP2R1*, *GC*, *CYP24A1*, *SEC23A*, and *AMDHD1*), an umbrella MR study in 339,256 White British participants

of the UK Biobank studied effects of 25(OH)D on hypertension and systolic and diastolic blood pressure, among other outcomes. Similar to the previous study, they failed to show any evidence for a causal association between genetically determined 25(OH)D levels and all three outcomes (all *P*-values >0.20) [56]. Finally, using up to 252 SNPs (applying SNP-filtering for pleiotropy) as instruments for 25(OH)D levels, a one-sample umbrella MR study [90], showed a marginal effect of 25(OH)D levels on risk of hypertension (MR OR 0.97, 95% CI 0.94–1.0) in 417,580 White British from UK Biobank. After adjusting for BMI, this estimate was no longer significant.

MR findings drawn in non-Europeans are consistent with those reported in European populations. Specifically, in 2581 Korean adults, MR failed to demonstrate any causal effect of 25(OH)D levels (inferred by four SNPs in *DHCR7*, *CYP2R1*, *GC*, and *CYP24A1*) on systolic and diastolic blood pressure and risk of hypertension (all *P*-values >0.45) [52]. Using four SNPs in the same genes, an MR study on 10,655 Chinese adults [54] showed equally a null effect of genetically determined 25(OH)D on systolic and diastolic blood pressure (MR estimate –0.20, 95% CI –3.0 to 2.6, and 0.06, 95% CI –1.78 to 1.90, respectively, per 10 nmol/L increase in 25(OH)D level).

In conclusion, all the aforementioned evidence does not support a causal role of 25(OH)D in hypertension in Europeans and Asians.

## 6. Musculoskeletal outcomes

There is strong evidence from MR studies showing no causal effect of vitamin D on a variety of bone traits in both Europeans and non-Europeans. The largest study in Europeans [24] reported that a genetically predicted 1 SD increase in 25(OH)D was not associated with higher femoral neck bone mineral density (BMD) (MR estimate 0.02, 95% CI –0.03–0.07), lumbar spine BMD (MR estimate 0.02, 95% CI –0.04–0.08), or estimated BMD (MR estimate –0.03, 95% CI –0.05 to –0.01). Similar results were observed in relation with total body BMD (MR estimate 0.92, 95% CI 0.82–1.04) [25]. Both studies performed a variety of sensitivity analyses, assessing heterogeneity and pleiotropy, after which the main effects remained unchanged. A large MR study on 37,857 fracture cases and 227,116 controls also did not support a causal effect of 25(OH)D on fracture risk (MR OR 0.84, 95% CI 0.70–1.02) [47], although an umbrella MR study showed a possible effect on leg and femur fractures [91]. Interestingly, a pediatric study showed that haplotypes associating with low 25(OH)D were also associated with low pQCT parameters (BMD, cross-

sectional area, and cortical density) in 2-year-old children [50], indicating that vitamin D may be important in this age group. More evidence from MR in children is needed to make strong inference of causality.

An MR study [19] using four SNPs, *GC*, *DHCR7*, *NADSYN1*, *CYP2R1*, and *CYP24A1* as instruments also did not provide evidence for causal associations between serum 25(OH)D and either BMD or bone metabolism markers in 1824 postmenopausal Chinese women. Per unit increase in log-transformed 25(OH)D, the MR effect was  $-0.048$  (95% CI  $-0.158$  to  $0.062$ ) for lumbar spine BMD,  $-0.044$  (95% CI  $-0.120$  to  $0.032$ ) for femoral neck BMD,  $-0.041$  ( $-0.123$ ,  $0.041$ ) for total hip BMD,  $0.088$  (95% CI  $-0.034$ – $0.210$ ) for parathormone (PTH), and  $0.099$  (95% CI  $0.291$ – $0.093$ ) for the amino-terminal propeptide of type 1 procollagen (P1NP).

Genetically determined higher serum 25(OH)D levels had a positive effect on total (MR estimate  $0.042$ , 95% CI  $0.005$ – $0.079$ ), trunk (MR estimate  $0.045$ , 95% CI  $0.006$ ,  $0.084$ ), and arm (MR estimate  $0.04$ , 95% CI  $0.015$ ,  $0.073$ ) fat-free mass; but not with leg fat-free mass (right leg MR estimate  $0.03$ , 95% CI  $-0.021$ ,  $0.081$ ; left leg effect  $0.039$ , 95% CI  $-0.006$  to  $0.084$ ) [89]. However, comprehensive and well-powered MR studies are needed to disentangle the effect of genetically determined 25(OH)D levels with muscle outcomes and traits. No MR studies so far have examined the causal association between genetically determined 25(OH)D levels and risk of falls.

## 7. Lung function and respiratory conditions

To date, there are no MR studies that have examined the effect of vitamin D on chronic obstructive pulmonary disease and lung function. There are only few MR studies that have investigated the causal association between vitamin D and asthma. An MR study on a small pediatric cohort [20] (5080 controls and 1203 cases) created an allelic score from two SNPs located near *GC* and *CYP2R1* and reported no association between genetically determined 25(OH)D concentrations on asthma (MR OR  $1.00$ , 95% CI  $0.996$ – $1.004$  or severe asthma exacerbations (MR OR  $0.99$ , 95% CI  $0.90$ – $1.09$ ). The findings were later confirmed by a study with relatively large study settings [36] and reported an MR OR of  $1.03$  (95% CI  $0.90$ – $1.19$ , 146,761 controls and 25,109 cases) for asthma and  $0.95$  (95% CI  $0.69$ – $1.31$ , 15,008 controls and 7047 cases) for childhood-onset asthma per standard deviation decrease in log-transformed 25(OH)D. Overall, the current studies do not support a causal association between 25(OH)D and the risk of asthma.

## 8. Immune disorders

There have been several MR studies examining the effect of 25(OH)D on a variety of immunological diseases. Currently, there is strong evidence supporting causal association between genetically low 25(OH)D levels and increased risk of multiple sclerosis (MS). The initial MR study in 2015 [15] used SNPs in/near *GC*, *DHCR7*, *CYP2R1*, and *CYP24A1* to infer causality between 25(OH)D levels and MS in Europeans and reported a 2.0-fold increase in the odds of MS (95% CI  $1.7$ – $2.5$ ) per 1 SD decrease in log-transformed 25(OH)D level. Recently, the findings were replicated in a larger and more powerful study [48], which included six loci (*CYP2R1*, *DHCR7*, *CYP24A1*, *SEC23A*, and *AMDHD1*) associated with 25(OH)D levels and reported a 75% increase in odds of MS (95% CI  $1.23$ – $2.44$ ) per one unit decrease in the natural log-transformed vitamin D level. Importantly, the study reported evidence for possible reverse causation between vitamin D and MS. The aforementioned results were replicated in at least two more recent MR studies: the first study [88], using 138 25(OH)D SNPs, showed a protective effect (MR OR  $0.82$ , 95% CI  $0.69$ – $0.99$ ) in a population of 14,498 MS cases and 24,091 controls. The second study [91] used 143 SNPs in an umbrella MR analysis in the UK Biobank and showed an MR OR for MS of  $0.96$  (95% CI  $0.94$ – $0.98$ ).

MR data do not support any association between 25(OH)D levels and systemic lupus erythematosus (MR OR  $1.03$ , 95% CI  $0.82$ – $1.303$ ) or rheumatoid arthritis (MR OR  $1.03$ , 95% CI  $0.91$ – $1.16$ ) [37] using three SNPs mapping to/near *SSTR4*, *GC*, and *NADSYN1* as instruments. A similar effect on rheumatoid arthritis was reported by the UK Biobank using  $\sim 220$  vitamin D-associated SNPs as instrumental variables (MR OR  $1.01$ , 95% CI  $0.80$ – $1.27$ ) [90]. A null effect was also reported for risk of juvenile idiopathic arthritis [70] in a sample of 14,872 cases and 12,501 controls. Similar results were obtained for risk of ankylosing spondylitis [71]. Recently, an MR study on 23,877 cases for knee osteoarthritis and 17,571 cases for hip osteoarthritis and using 6 25(OH)D SNPs, found no effect of 25(OH)D for any of the two outcomes [72].

Vitamin D levels have also been shown not to be causally associated with Crohn's disease and ulcerative colitis. Per 10 nmol/L higher genetically determined 25(OH)D levels, the ORs were  $0.88$  (95% CI  $0.64$ – $1.21$ ) for Crohn's disease and  $1.04$  (95% CI  $0.82$ – $1.32$ ) for ulcerative colitis [39]. Similarly, no effect on ulcerative colitis was reported by the UK Biobank using  $\sim 270$  vitamin D-associated SNPs as instrumental variables (MR OR  $0.99$ , 95% CI  $0.79$ – $1.26$ ) [90]. This one-sample MR study in UK Biobank did support a causal role of

vitamin D on allergic rhinitis (MR OR 1.04, 95% CI 1.01–1.10). Also, MR failed to show a causal effect on genetically estimated 25(OH)D on atopic dermatitis (MR OR 1.12, 95% CI 0.92–1.37) [36], while a more recent study showed null effects on allergic and nonallergic rhinitis and risk of allergic sensitization [73].

The only available MR studying the effects of vitamin D on type 1 diabetes [74] used 69 common 25(OH)D SNPs and did not show a significant effect of vitamin D levels in a cohort of 9358 cases and 15,705 controls of European ancestry (MR OR 1.09, 95% CI 0.86–1.40, per 1-SD decrease in standardized log-transformed 25(OH)D). Finally, a metaanalysis of MR studies on a total of 2214 cases and 5695 controls from Asian and European background found a significant effect of 25(OH)D levels (MR OR 3.96, 95% CI 1.72–9.13) on risk of Behcet's disease [75].

## 9. Pregnancy-related outcomes

To date, only one MR study [38] has examined the causal effect of 25(OH)D serum levels on pregnancy-related hypertensive disorders such as gestational hypertension and preeclampsia, using vitamin D synthesis and metabolism SNPs. In total, the study included 7389 women in a one-sample MR analysis (751 with gestational hypertension and 135 with preeclampsia), and 3388 preeclampsia cases and 6059 controls in a two-sample MR analysis. There was no strong evidence to support a causal effect of vitamin D status on gestational hypertension (MR OR 0.90, 95% CI 0.78–1.03) or preeclampsia (MR OR 0.98, 95% CI 0.89–1.07). Another MR study did not find any association between the maternal vitamin D status and the first child's birthweight in UK Biobank (MR estimate [difference in mean birth weight] –0.03 g, 95% CI –2.48 to 2.42 g per 10% higher maternal 25(OH)D) [60].

## 10. Neuropsychiatric outcomes

MR has been used to study the role of vitamin D on several neuropsychiatric outcomes, such as depression, attention-deficit/hyperactivity disorder (ADHD), and Alzheimer's disease (AD). For instance, it has been reported that genetically increased 25(OH)D levels are associated with reduced AD risk [16,40,51]. However, a recent large-scale MR study using 138 SNPs failed to corroborate the beneficial effect of 25(OH)D levels on AD risk [97]. Further, MR showed no clear support that genetically determined 25(OH)D levels are causally associated with risk of Parkinson's disease (MR OR 0.98,

95% CI 0.93–1.04) [34] nor of depression (MR OR 0.97, 95% CI 0.90–1.05) [64]. This corroborates the findings from previous MR studies [41,57,61]. Similarly, MR did not support a causal relationship between 25(OH)D levels in studies investigating other forms, such as treatment-resistant depression in 1891 cases (MR OR 1.01, 95% CI 0.78–1.31) or atypical depression in 2101 cases (MR OR 1.04, 95% CI 0.80–1.36) [80]. In contrast, using a bidirectional MR analysis, genetic predisposition to depression was associated with lower 25(OH)D concentrations [64]. There is also little evidence for a causal effect of 25(OH)D on fatigue (MR OR 1.05, 95% CI 0.87–1.27, per 1-SD decrease in log-transformed 25(OH)D) [32]. Furthermore, it has been shown that participants carrying more vitamin D-increasing variants have reduced likelihood of incident delirium diagnosis (HR 0.80, 95% CI 0.73–0.87, per 1-SD increase in genetically determined vitamin D) [78]. MR also suggests no evidence for a causal effect of 25(OH)D on ADHD risk [79] or amyotrophic lateral sclerosis [53].

## 11. COVID-19

Given the recent advent of observational studies suggesting that vitamin D supplementation could modify the risk of COVID-19-related outcomes, MR studies were launched to assess the causal role of 25(OH)D levels on COVID-19 susceptibility and disease severity. Using data from 11,181 cases and 116,456 controls from the Host Genetics Initiative, and 17 vitamin D SNPs explaining 2.5% of the variance in 25(OH)D levels, this study [83] did not show any evidence for association between genetically decreased 25(OH)D and COVID-19 outcomes (MR OR 1.04, 95% CI 0.91–1.03, for COVID-19 infection, and MR OR 0.79, 95% CI 0.58–1.08, for severe COVID-19 infection). These results were confirmed by a separate MR study [82] using 81 25(OH)D SNPs explaining 4.3% of the variance in 25(OH)D levels, which also showed no impact of genetically determined 25(OH)D levels on risk of COVID-19-related hospitalization (MR OR 0.95, 95% CI 0.84–1.08 for COVID-19 risk, MR OR 1.09; 95% CI 0.89–1.33 for hospitalization, and MR OR 0.97, 95% CI 0.77–1.22 for severe COVID-19 infection).

## 12. Mortality risk and aging

In a large-scale study on mortality risk (comprising 10,349 deaths in 95,766 participants) [98], genetic variants in the vitamin D synthesis pathway mapping to *DHCR7* and *CYP2R1* were used as instruments to infer



25(OH)D levels. The MR OR for a genetically determined 20 nmol/L lower 25(OH)D concentration was 1.30 (95% CI 1.05–1.61) for all-cause mortality, 0.77 (95% CI 0.55–1.08) for cardiovascular mortality, 1.43 (95% CI 1.02–1.99) for cancer mortality, and 1.44 (95% CI 1.01–2.04) for other mortality. Similar nonsignificant effect sizes were reported [28] per 20 nmol/L decrease in genetically determined 25(OH)D levels, specifically 1.32 (95% CI 0.80–2.24) for all-cause mortality in a combined sample from three European cohort studies (10,501 participants and 4003 deaths). This study may have been underpowered to detect existing causal associations with mortality. In addition, no causal effect (MR OR 1.09, 95% CI 0.79–1.50) was reported [56] using six independent SNPs in *GC*, *NADSYN1/DHCR7*, *CYP2R1*, *CYP24A1*, *AMDHD1*, and *SEC23A*. This study reported having 85% power to detect an OR of 1.2; thus, it may have not been powered to detect possible smaller effects. Finally, no significant association was found between 25(OH)D levels and cancer mortality (MR OR 0.97, 95% CI 0.84–1.11, in 6998 deaths) [27]. Importantly, recent MR studies have demonstrated a nonlinear causal effect on all-cause mortality (discussed in the following). MR also showed a null effect on telomer shortening (MR estimate  $-0.10$ , 95% CI  $-0.27$  to  $0.06$ ), often considering proxy of human aging [49].

### 13. Other outcomes

Several other disease-related outcomes have been subject of scrutiny for a relationship with vitamin D levels using the MR approach. For every 1 SD increase in genetically determined 25(OH)D, the combined MR OR of NAFLD was 0.78 (95% CI 0.69–0.89). Genetically predicted higher levels of 25(OH)D showed a suggestive association with aspartate aminotransferase levels (MR estimate  $-0.17$ , 95% CI  $-0.36$  to  $0.01$ ) [87]. Genetic predisposition to a 1 SD higher 25(OH)D level was associated with increased serum calcium levels (MR estimate 0.014, 95% CI 0.010–0.018) and increased risk of kidney stones (MR OR 1.47, 95% CI 1.22–1.77) [85]. Furthermore, MR reported a negative causal effect of log-transformed 25(OH)D on log-transformed eGFR (MR estimate  $-0.013$ , 95% CI  $-0.002$  to  $-0.005$ ) [35]. MR did not provide evidence of a causal effect of vitamin D on concentrations of C-reactive protein (MR estimate  $-0.001$ , 95% CI  $-0.007$  to  $0.005$ ),  $\alpha$ 1-acid glycoprotein (MR estimate  $-0.001$ , 95% CI  $-0.008$  to  $0.006$ ), and soluble intercellular adhesion molecule 1 (MR estimate  $-0.002$ , 95% CI  $-0.009$  to  $0.005$ ) [65]; all considered relevant inflammatory biomarkers. MR showed a suggestive association between 1-SD increase in genetically determined 25(OH)D and lower levels of cholesterol, lipoprotein particles, and phospholipids within

very-small very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) [92]. Per 1-SD increase in genetically determined 25(OH)D, there was 0.12 units decrease in total testosterone in 4254 Chinese men (95% CI, 0.02–0.22) [23]. On the other hand, a null MR effect of 25(OH)D was shown on circulating insulin-like growth factor-binding protein 3 (IGFBP-3) levels [43].

A potential causal association of serum 25(OH)D with number of remaining natural permanent teeth (MR estimate 0.085, 95% CI 0.019–0.150) was reported [86] but not with dental caries or periodontitis. Two additional MR studies have also reported a null effect on caries [77] and periodontitis risk [81]. Genetically determined 25(OH)D levels are reported to not have an effect on refractive error (MR estimate  $-0.02$ , 95% CI  $-0.09$  to  $0.04$  diopters per 10 nmol/L increase in 25(OH)D concentration in Caucasians and 0.01 95% CI  $-0.17$  to  $0.19$  diopters per 10 nmol/L increase in Asians) [30]. Also, MR showed no causal association between 25(OH)D and features of skin aging [44].

### 14. Nonlinear MR studies

Taking advantage of the availability of data on 25(OH)D levels and a large spectrum of phenotypes in UKBB and other large-scale genotyped cohorts, the MR design was recently adapted to study nonlinear effects. To do this, one-sample MR studies have been undertaken in strata of the studied populations, specifically in individuals presenting deficient, insufficient, sufficient, and adequate 25(OH)D levels ( $<25$  nmol/L, 25–50 nmol/L, 50–75 nmol/L, and  $>75$  nmol/L, respectively). A stratified MR study [99] investigated the effect of 25(OH)D on cardiovascular disease (CVD), ischemic stroke, and all-cause mortality using 71 SNPs, explaining up to 4.7% of the variance in 25(OH)D. The study population comprised 386,406 middle-aged cases with CVD, 18,166 people who had a stroke, and 27,885 people who died. MR analyses showed in individuals with vitamin D deficiency an inverse association with all-cause mortality (OR per 10 nmol/L increase in genetically predicted 25(OH)D concentration 0.69 (95% CI 0.59–0.80) and nonsignificant inverse associations with stroke (MR OR 0.85, 95% CI 0.70–1.02) and CVD (MR OR 0.89, 95% CI 0.76–1.04). Another nonlinear MR study [100] used 35 SNPs explaining 2.8% of the variance in 25(OH)D levels in a population of 44,591 CVD cases and 251,269 controls of the UKBB. They showed an L-shaped association between genetically predicted serum 25(OH)D and CVD risk ( $P = .007$ ), and a similar association for systolic blood pressure ( $P = .03$ ) and a trend for diastolic blood pressure ( $P = .07$ ).

## 15. Conclusion

Improved understanding of the genetic determinants of 25(OH)D has helped reassess the role of vitamin D in the etiology of complex traits and diseases through MR. Taken together, the evidence from over 100 available vitamin D MR studies does not support a causal role for the vast majority of studied health outcomes. Nonetheless, in the few cases where the evidence from MR supported causality of vitamin D, such as in the example of MS, these findings have important clinical implications. For instance, recent clinical care guidelines published by the MS Society of Canada recommend the use of vitamin D in preventing multiple sclerosis in those at increased risk [101]. Early MR studies used as instruments for 25(OH)D serum levels SNPs within the four genes related to 25(OH)D synthesis and metabolism (*DHCR7*, *CYP2R1*, *GC*, and *CYP24A1*), which together explained 2.4% of the variance in 25(OH)D levels [102]. Subsequent MR studies combined the aforementioned four SNPs with two additional SNPs mapping to *SEC23A* and *AMDHD1* (both genes without clear role in the vitamin D metabolic pathway), explaining together ~5.3% of the variance of 25(OH)D levels. The recent identification of over 150 25(OH)D-associated genetic variants, which explain a significantly larger portion of the variance in 25(OH)D levels (~10.5%) [90], has allowed a deeper understanding of the genetic determinants contributing to variation in circulating 25(OH)D levels and has enabled an improved instrumentation of vitamin D in MR studies.

The MR results corroborate with the data generated by recent megatrials of vitamin D supplementation [7–10] of largely vitamin D-sufficient adults, demonstrating that increasing serum 25(OH)D concentration into the high normal range (based on the Institute of Medicine and most recent guidelines in the range of 50–125 nmol/L or 20–50 ng/mL) does not generate benefits for global health or major diseases such as cancer, cardiovascular events, T2D, or fractures. Therefore, there is no reason at present to recommend vitamin D supplementation in already vitamin D replete subjects. This contention does not contradict the causal link between severe vitamin D deficiency and rickets and the need to correct (severe) deficiency at any age. In this line, findings from recent nonlinear MR studies support the need to correct vitamin D deficiency to prevent CVD and all-cause mortality, which is in line with previous evidence from RCTs. Further, evidence from nonlinear MR studies can elucidate causal associations of 25(OH)D levels with diseases and traits in individuals with 25(OH)D in the extremes of the normal distribution. These dose-dependent effects of 25(OH)D levels need to be also evaluated in well-designed RCTs.

The available evidence from vitamin D MR studies is largely concordant with that from RCTs. While these findings advocate correction of vitamin D deficiency, they do not argue in favor of systematically recommending vitamin D supplementation in replete individuals for a wide range of common diseases. With the emergence of large-scale GWAS on densely phenotyped biobanks, we anticipate that more powerful vitamin D MR studies will be published, utilizing optimized sets of genetic instruments, revisiting previously studied diseases, investigating new disease outcomes, and assessing nonlinear effects, to further aid causal effect estimation.

## 16. Summary points

- Over 100 MR studies have evaluated the consequences of lifelong genetically determined serum levels of 25(OH)D on various outcomes, and most studies have observed null effects.
- A notable exception are four independent MR studies showing a higher risk of multiple sclerosis in subjects with genetically lowered 25(OH)D.
- MR studies evaluating nonlinear effects have shown evidence for causal effects of low serum 25(OH)D (<50 nmol/L) on cardiovascular disease and all-cause mortality.
- Indiscriminate vitamin D replacement in the general population is unlikely to be helpful, except for the prevention of multiple sclerosis.
- Vitamin D replacement is likely beneficial for those with established vitamin D deficiency.

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## Further reading

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# Growth plate histology, bone histomorphometry, and radiologic features of nutritional rickets and osteomalacia

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## OBJECTIVES

- Review the terminologies related to bone formation and mineralization.
- Understand the normal anatomy and the pathophysiology of rickets at the growth plate.
- Review the clinical and radiological features of rickets and osteomalacia.
- Review the differentiating classic radiological features of various forms of calcipenic and phosphopenic rickets.
- Review the histomorphometric features and bone mineralization density distribution curve on backscattered electron imaging in osteomalacia.
- Review the utility of other imaging modalities such as MRI and DXA scans in the diagnosis of hypomineralization disorders.

## 1. Introduction

Rickets results from defective mineralization of the growth plate and is seen in growing children. Osteomalacia, on the other hand, results from reduced mineralization of the preformed osteoid and can be seen in both adults and children. Rickets and osteomalacia can result

from lack of minerals (calcium and/or phosphate), mineral suppliers [calcitriol or 1,25 dihydroxyvitamin D (1,25(OH)<sub>2</sub>D)], or facilitators of mineralization [such as alkaline phosphatase (ALP)]. Several genetic causes of rickets and osteomalacia have been described; nonetheless, nutritional deficiencies in dietary calcium and/or vitamin D remain the leading causes worldwide [1,2]. The main source of vitamin D is cutaneous synthesis following skin exposure to ultraviolet B (UVB) rays in sunlight. Therefore, vitamin D deficiency (low serum 25-hydroxyvitamin D, 25(OH)D) is more commonly seen in high-latitude countries and predominantly in dark-skinned populations or in individuals adapting culturally covered clothing. The main source of calcium, however, is diet, and therefore, calcium deficiency is most commonly seen along with other nutritional deficiencies and predominates in the undernourished populations of low- to middle-income countries [3].

Rickets is a radiological diagnosis reflecting the histopathological changes of defective mineralization at the growth plate and adjacent metaphysis of long bones. Osteomalacia is not a radiological diagnosis but diagnosed on histomorphometry of bone biopsy, and reflects defective mineralization of the preformed osteoid. Both conditions, however, have typical clinical and biochemical features that aid in diagnosis. Since calcium and vitamin D deficiency are widespread micronutrient deficiencies, with serious health consequences, they represent a major global public health concern and require simple and cost-effective prevention strategies.

## 2. Terminology related to bone formation and mineralization

The terminologies relating to bone formation and mineralization are detailed in [Table 62.1](#).

**TABLE 62.1** Terminology relating to bone formation and mineralization.

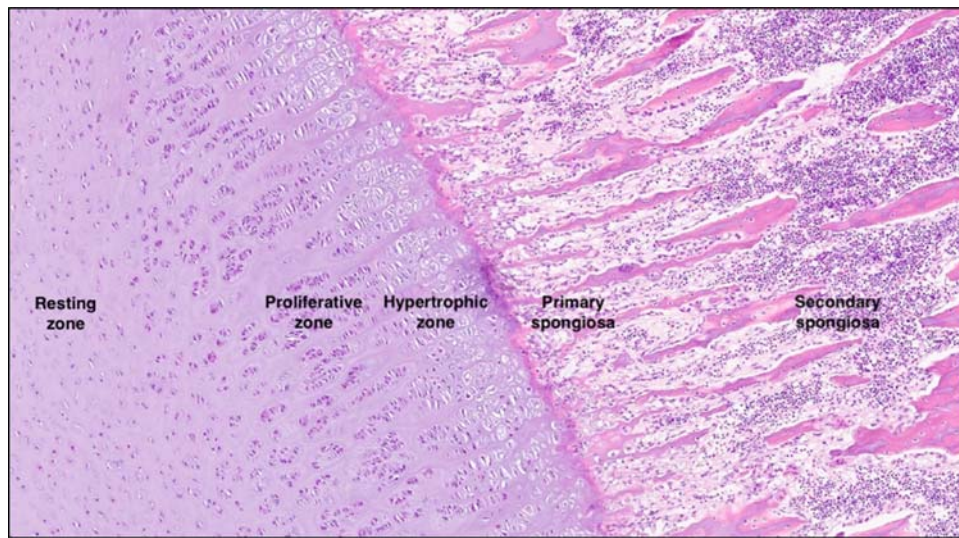
Terminology	Clarification
Bone formation	Describes the process of synthesis of extracellular organic osteoid followed by its mineralization.
Ossification	Relates to the two major modes of bone development [4]: - <b>Endochondral ossification</b> seen in long bones (e.g., femur) where cartilage is replaced by bone tissue (discussed in detail later). - <b>Intramembranous ossification</b> seen in flat bones (e.g., skull). In intramembranous ossification, mesenchymal cells differentiate directly into osteoblasts, which in turn synthesize bone matrix. Unlike cartilage, bone is incapable of internal expansion, and therefore, the growth of bones formed by intramembranous ossification must occur through surface apposition.
Mineralization	Relates to the integration of hydroxyapatite [ $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ] into osteoid.
Calcification or pathological mineralization	Deposition of calcium phosphate crystals in extraskeletal tissues (kidneys, skin, arteries, muscle, brain, tumors) [5].
Bone modeling	Process where bones are shaped or reshaped by the independent action of osteoblast and osteoclasts. Bone modeling defines skeletal development and growth but continues throughout life [6].
Bone remodeling	Process where osteoclasts and osteoblasts work sequentially in the same bone remodeling unit. The human skeleton is renewed by remodeling throughout life [6].
Rickets	Defective mineralization of the growth plate cartilage and adjacent primary and secondary spongiosa in the metaphysis (“new bone”).
Osteomalacia	Defective mineralization of newly formed osteoid in existing bone during remodeling (“old bone”).
Osteoporosis	Reduced bone mass and structural quality. It is not associated with reduced mineralization of bone tissue. Osteoporosis occurs when bone resorption exceeds bone formation (adults and children) or from reduced bone mass accrual (children).

## 3. The growth plate

The growth plate, wedged between the metaphysis and the epiphysis of long bones, is the key player in promoting long bone growth in children. Endochondral bone formation is the fundamental mechanism of bone growth. During endochondral ossification, the growth plate undergoes morphogenesis, and cartilage is replaced by bone. The process of ossification begins with the condensation of undifferentiated mesenchymal cells [7]. The cellular components of this condensation differentiate into chondrocytes that lay down the cartilage matrix, which is eventually replaced by bone [7]. The growth plate therefore is predominantly composed of cartilage and chondrocytes, which are arranged in distinctive layers. The three principal chondrocyte zones in the growth plate that contribute to longitudinal growth in the postnatal period are the resting, proliferative, and hypertrophic zones ([Fig. 62.1](#)).

The resting zone, adjacent to the epiphyseal bone, maintains the integrity of the growth plate structure, particularly by expressing parathyroid hormone related protein (PTHrP) [8]. The resting zone contains round chondrocytes embedded within the cartilaginous matrix, and as the cells begin to divide, the proliferative zone is formed [8]. The chondrocytes in the proliferative zone are arranged in columns parallel to the long axis of the bone, and the round proliferating chondrocytes become flattened as they are packed into multicellular columns. The proliferative chondrocytes farthest from the resting zone stop replicating and enlarge in size significantly to become hypertrophic chondrocytes [9]. The cells in the transition zone from proliferative to hypertrophic are also recognized as the “prehypertrophic” chondrocytes. The rate at which proliferating chondrocytes undergo hypertrophy determines bone elongation [10] with enlargement of the hypertrophic chondrocytes being the major contributing factor regulating the growth rate in endochondral bones. This metric is largely responsible for the variations in skeletal growth rates among different endochondral bones within an individual [11]. The hypertrophic chondrocytes produce a matrix rich in collagen X as well as angiogenic factors such as vascular endothelial growth factor (VEGF) [12] that attract blood vessels and bone cells. Hypertrophic chondrocytes express the molecular signals that direct mineralization of the surrounding matrix, such as the Indian hedgehog (Ihh). The terminally differentiated chondrocytes mineralize the cartilage matrix to form the zone of provisional calcification. The hypertrophic chondrocytes undergo apoptosis or differentiate into osteoblasts to form a bony matrix known as the primary spongiosa, which is the future site of trabecular bone [13].





**FIGURE 62.1** Normal human growth plate at the rib costochondral junction. Illustrates H&E stained ( $\times 4$  magnification) growth plate histology demonstrating normal growth plate with distinct chondrocyte zones (resting, proliferative, and hypertrophic) and spongiosa (primary and secondary).

The calcified cartilage matrix produced in the growth plate provides the template for the production of primary spongiosa (primary trabeculae). The osteoclasts (bone resorbing cells) remove cartilage matrix, and osteoblasts use the remnants of cartilage matrix as a scaffold for the deposition of bone matrix, thereby replacing the calcified cartilage with new (or secondary) trabecular bone resulting in remodeling into mature secondary spongiosa. Thus, during growth, there is a dynamic transformation of growth plate cartilage into new trabecular bone wherein growth plate matrix structure and bone trabecular structure are intimately associated having separate but complementary functions in achieving overall skeletal development. Overall, bone growth and development is tightly regulated by both systemic factors and locally secreted factors, which act on receptors to effect intracellular signaling and activation of chondrocyte-selective transcription factors, which are discussed in detail elsewhere [7,11,14].

### 3.1 The growth plate in rickets

The basic pathology underlying both phosphopenic and calcipenic (secondary hyperparathyroidism leading to renal phosphate wasting and subsequent hypophosphatemia) forms of rickets is the lack of phosphate at the growth plate [15]. Phosphate is essential for apoptosis of the hypertrophic chondrocytes and the mineralization that follows. Phosphate-regulated apoptosis of hypertrophic chondrocytes is activated via the caspase-9-mediated mitochondrial pathway [16]. Lack of apoptosis leads to accumulation of

hypertrophic chondrocytes, which leads to an expansion of the hypertrophic zone as the chondrocytes, previously arranged in columns, become disarrayed. Mice models with varying etiologies of low phosphate such as vitamin D receptor mutation, diet-induced hypophosphatemia/hypercalcemia (with suppressed parathyroid hormone), and hypophosphatemia secondary to mutations in the PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) gene (with normal calcium and parathyroid hormone levels, but inappropriately low 1,25-dihydroxyvitamin D levels) demonstrate changes of rickets at the growth plate [16]. Calcium-sensing receptor knockout mice also exhibit classical rachitic growth plates secondary to hyperparathyroidism (due to impaired parathyroid calcium sensing which leads to hypophosphatemia) supporting the hypothesis that phosphate is a key regulator of chondrocyte apoptosis [17]. Similarly, vitamin D receptor (VDR) null mice demonstrate rickets at growth plate and skeletal demineralization due to impaired intestinal calcium absorption and the resultant secondary hyperparathyroidism and hypophosphatemia [18]. The skeletal phenotype of the VDR null mice is reversed with introduction of a rescue diet enriched with calcium, phosphorus, and lactose indicating the rachitic changes are a direct result of deficient mineral supply at the growth plate, and not a direct effect of vitamin D [18].

The histological changes of rickets at the growth plate include [19] expansion and disarray of the hypertrophic zone due to accumulation of hypertrophic chondrocytes, lack of clear distinction between the various growth plate zones and fading of the zone of provisional

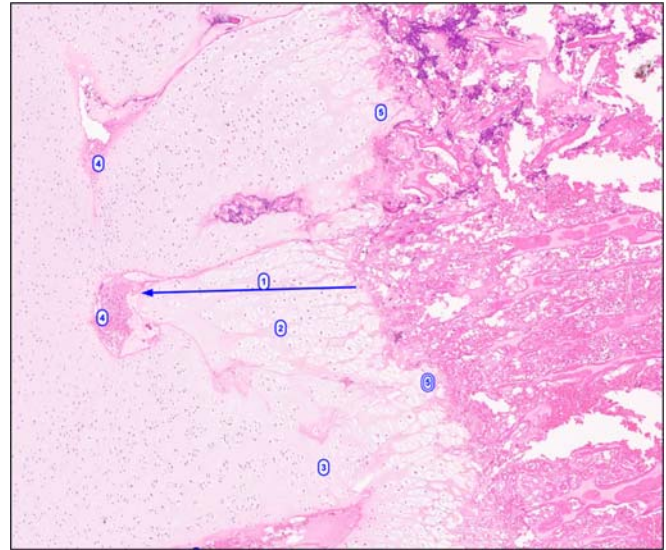
calcification, penetrating blood vessels in the hypertrophic chondrocyte cartilage zone, and tongue-like projections of cartilage extending into the primary spongiosa (Fig. 62.2A). The expansion of the hypertrophic zone leads to a bulbous appearance of the growth plate histologically (Fig. 62.2B).

In severe cases of rickets, the enlarged hypertrophied chondrocyte layer contributes to the clinical appearance of swollen joints at the wrist and/or ankle (Fig. 62.3A) and the corresponding radiological appearance of the widened metaphyses (Fig. 62.3B). Detailed radiological features of rickets are discussed in the radiology section. Bulbous expansion of the costochondral junction manifests as rachitic rosary or beaded ribs (Fig. 62.4).

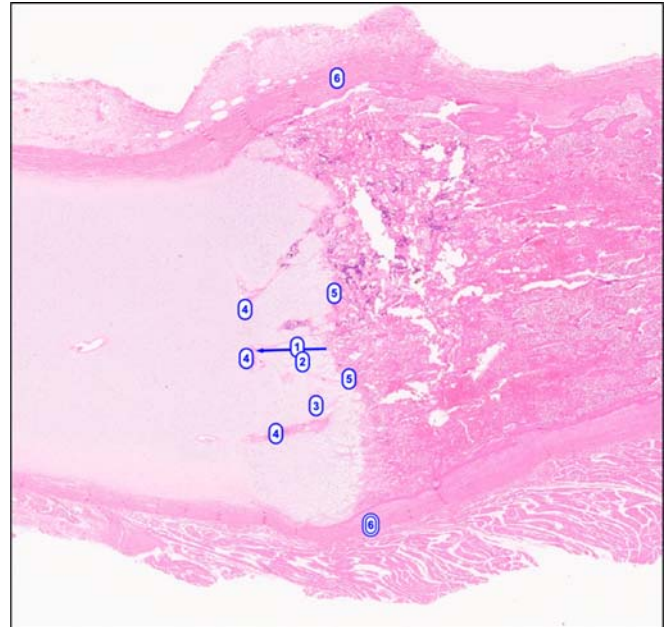
#### 4. Mineralization

Bone is mineralized connective tissue composed of 50%–70% mineral with the most abundant minerals being calcium and phosphorus, present in the form of hydroxyapatite crystals  $[\text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2]$  [4]. The mineralization process therefore requires adequate substrates, which are supplied by  $1,25(\text{OH})_2\text{D}$  through intestinal absorption. Additionally, tissue-nonspecific alkaline phosphatase (TNSALP) also plays a crucial role in the formation of hydroxyapatite crystals, and reduced activity leads to hypomineralization as seen in hypophosphatasia [20].

Several theories for the mechanism of initiation and regulation of cell-mediated mineralization have been proposed [21]. While it is not entirely clear as to what initiates mineralization, it is, however, well recognized that matrix vesicles (MVs) protruding from osteoblasts play an important role [22]. The first step in mineralization is the formation of hydroxyapatite in MVs of mature chondrocytes and osteoblasts [23]. In brief, during phase I, there is increased activity of the MV phosphatases (including ALP, adenosine triphosphatase, pyrophosphatase, and PHOSPHO1), which generate and transport phosphate, as well as calcium-binding compounds such as the annexin family and phosphatidylserine [24]. Calcium and phosphate are attracted into the MVs by these compounds, until the threshold for their precipitation is reached [22]. In phase II, the hydroxyapatite crystals penetrate the MV membrane and are elongated into the extracellular space [22], which requires appropriate concentrations of calcium and phosphate outside the MVs. TNSALP hydrolyses inorganic pyrophosphate (PPi), an inhibitor of hydroxyapatite formation, to inorganic phosphorus (Pi), thereby facilitating hydroxyapatite crystal formation [25]. The ratio of PPi to Pi is considered critical in the promotion or restriction of mineral in physiological tissues [23]. TNSALP, encoded by the *ALPL* gene, is found in abundance on the surface of MVs [26].

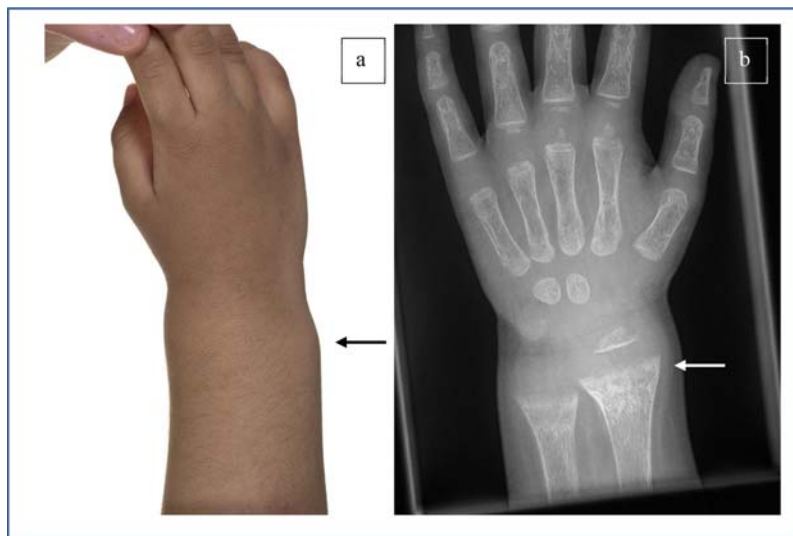


**FIGURE 62.2A** Changes of severe rickets on growth plate histology. Illustrates H&E stained ( $\times 4$  magnification) growth plate histology demonstrating significant histological changes of rickets. Arrow demonstrates the increased height of the hypertrophic zone and 1 = expansion of the hypertrophic zone, 2 = disarrayed hypertrophic chondrocytes, 3 = loss of distinction between hypertrophic and proliferative zones, 4 = blood vessels penetrating the chondrocytes, 5 = tongues of cartilage extending into primary spongiosa.

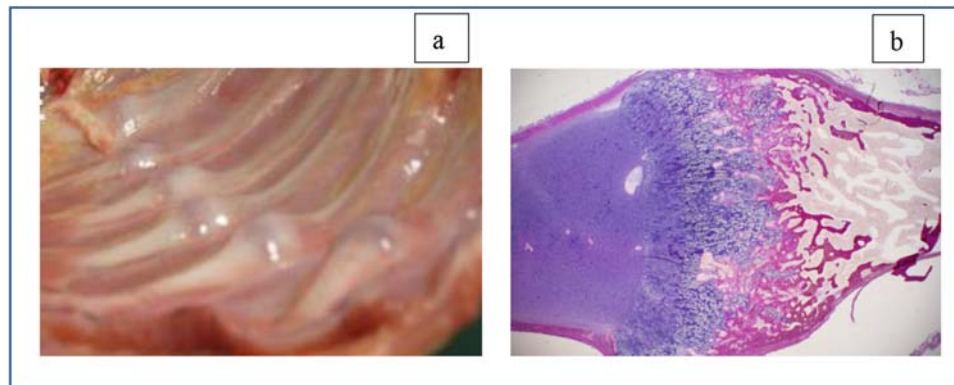


**FIGURE 62.2B** Changes of severe rickets on qualitative growth plate histology demonstrating bulbous expansion of the costochondral junction. Illustrates H&E stained ( $\times 1$  magnification) growth plate histology demonstrating significant histological changes of rickets. Arrow demonstrates height of the hypertrophic zone and 1 = expansion of the hypertrophic zone, 2 = disarrayed hypertrophic chondrocytes, 3 = loss of distinction between hypertrophic and proliferative zones, 4 = blood vessels penetrating the chondrocytes, 5 = tongues of cartilage extending into primary spongiosa, and 6 = bulbous expansion of the costochondral junction.





**FIGURE 62.3** (A) demonstrates swollen wrist joint in a 3.5-year-old child with severe nutritional rickets who presented with regression of motor development milestones (adjusted calcium 1.63 mmol/L, phosphate 0.7 mmol/L, ALP 1776 IU/L, 25 hydroxyvitamin D < 7.5 nmol/L, and parathyroid hormone 159 pmol/L (reference range 1.6–7.5)). (B) Illustrates the corresponding radiograph demonstrating widening of the metaphyses.



**FIGURE 62.4** (A) demonstrates rachitic rosary at postmortem examination in a 6-month-old child with severe nutritional rickets (adjusted calcium 1.6 mmol/L, ALP 802 IU/L, 25 hydroxy vitamin D < 15 nmol/L, parathyroid hormone 167 ng/L (reference range 12–29)) who presented in cardiac arrest due to hypocalcemic dilated cardiomyopathy. (B) Illustrates the corresponding histological section ( $\times 1$  magnification) of the seventh rib (Elastica van Gieson staining) demonstrating expansion and disarray of the hypertrophic zone.

## 5. Bone histomorphometry in osteomalacia

Histomorphometry is the quantitative evaluation of bone tissue including both the activity of bone cells and the amount and distribution of bone tissue. Histomorphometry enables the assessment of bone structure and the dynamics of bone modeling and remodeling through the administration of time-spaced two oral doses of tetracycline derivative prebiopsy. Osteomalacia is a histological diagnosis and is usually accompanied by typical biochemical derangements of serum ALP, calcium, and/or phosphate. However, it can sometimes exist in the absence of these biochemical abnormalities [27].

In osteomalacia, old bone resorbed during remodeling is replaced by unmineralized bone matrix (or osteoid tissue). Histological differentiation of osteomalacia from osteoporosis and osteitis fibrosa was first made by the

German pathologist Pommer in the late 19th century, which was subsequently elaborated by Parfitt based on the current concepts of bone remodeling [28]. Tetracyclines bind to newly calcified bone, which fluoresces under UV light. This characteristic is used to assess bone formation after giving oral tetracycline on two occasions (double labeling) before obtaining an iliac-crest biopsy. Based on tetracycline-labeled histomorphometry studies, Parfitt classified hypovitaminosis D osteopathy (HVO) into three stages [28].

- HVOi: The earliest stage of HVO where there is osteoid accumulation, before the emergence of a significant mineralization defect, because of increased frequency of remodeling activation and bone turnover due to secondary hyperparathyroidism. This state is also referred to as preosteomalacia.

- HVOii: The stage of defective mineralization where there is some retention of tetracycline double labels.
- HVOiii: The advanced stage of defective mineralization where tetracycline labels are not retained.

HVOi is characterized by an increase mainly in osteoid surface with a slight increase in osteoid thickness (usually  $<12.5\ \mu\text{m}$ ) [29]. In individuals with vitamin D deficiency, there is a hyperbolic relationship between osteoid surface and osteoid thickness, meaning that osteoid surface increasing first, and osteoid thicknesses increasing only when osteoid surface exceeds  $>70\%$  of the total bone surface [29]. Thereafter, any further increase in osteoid surface is accompanied by a substantial increase in osteoid thickness and osteoid volume. Additionally, a mineralization lag time, an index of the time interval between matrix apposition and its subsequent mineralization, of  $>100$  days separates patients with and without osteomalacia [29]. Histologic osteomalacia in adults is generally defined by a combination of osteoid thickness  $>12.5\ \mu\text{m}$  and a mineralization lag time of  $>100$  days [29]. Mineral apposition rate (MAR) can also be reduced in osteomalacia, but it is not specific to osteomalacia. Cortical thinning and bone marrow fibrosis occurring as a consequence of secondary hyperparathyroidism are additional features of vitamin D deficiency osteomalacia.

While the causes and histomorphometric features of osteomalacia are similar between adults and children, there is no specific definition of osteomalacia in children. There are no histomorphometry reference data from children under 1.5 years of age. In children aged 1.5–22.9 years ( $n = 58$ ), reference histomorphometry parameters are reported to be dynamic with osteoid surface relative to bone surface (OS/BS) and osteoid volume relative to bone volume (OV/BV) being naturally higher in the younger age groups demonstrating a steady decrease with age similar to the MAR [30]. The osteoid thickness,

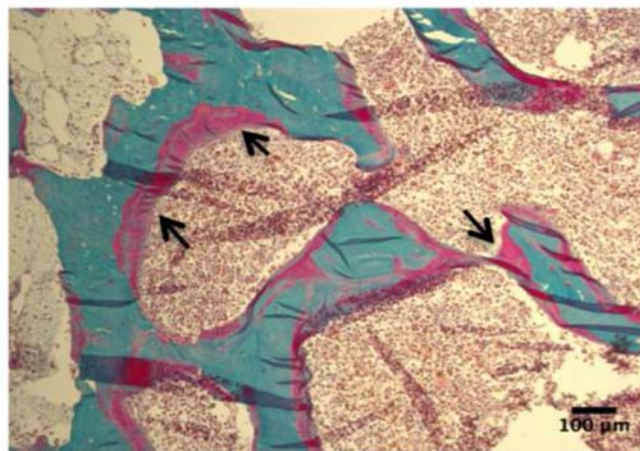
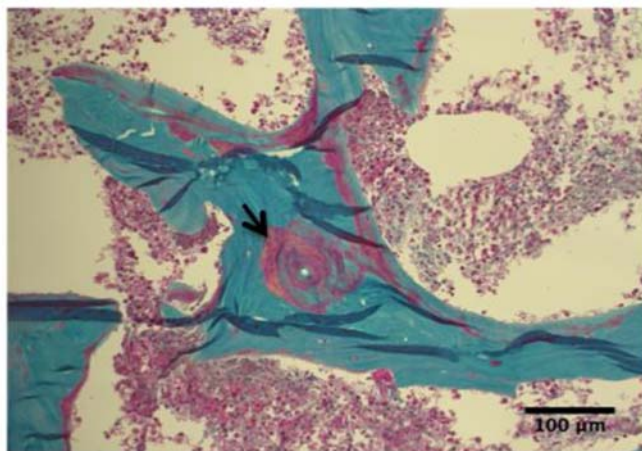
however, is reported to be stable with age [30]. Fig. 62.5 illustrates Goldner's Trichrome stained iliac bone section of an infant who died of dilated cardiomyopathy and heart failure due to severe vitamin D deficiency [31]. Significant osteomalacia (Fig. 62.5) is noted with increased osteoid thickness ( $23.2\ \mu\text{m}$  [reference  $6.4 \pm 1.4$ ]), OS/BS ( $76.3\%$  [reference  $24.9 \pm 10$ ]), and OV/BV ( $40.5\%$  [reference  $2.4 \pm 1.22$ ]) when compared with normative data from older children (Fig. 62.5) [30].

A study of bone histomorphometry in Black African adolescents with dietary calcium deficiency ( $n = 26$ ) demonstrated an inverse correlation between serum calcium levels and eroded mineralized surface, osteoid surface, osteoid thickness, mineralization lag time, and  $1,25(\text{OH})_2\text{D}$  [32]. The cortical bone in calcium deficiency also displays features of hyperosteoridosis, and increased erosion and porosis secondary to hyperparathyroidism [33]. Children with calcium deficiency are reported to display osteomalacia even in the absence of radiological signs of rickets; however, most of these were adolescents where signs of rickets may not be evident due to closure of the growth plate [33]. Osteomalacia solely due to calcium deficiency in adults has not been assessed or reported to date [29].

In hypophosphatemic osteomalacia, the degree of osteoid accumulation, both osteoid surface and thickness, is reported to be much greater than in vitamin D deficiency osteomalacia [29,34,35]. Furthermore, the osteoid indices return to normal after successful therapy in vitamin D deficiency osteomalacia [36], but not in X-linked hypophosphatemic osteomalacia [34,37].

## 6. Bone mineral density distribution in osteomalacia

Bone strength is determined by its intrinsic material properties, and therefore the mineral content of the bone matrix plays a critical role in determining bone



**FIGURE 62.5** Goldner's Trichrome staining (light microscopy) demonstrating broad seams of pink stained areas corresponding to non- or poorly mineralized matrix and regions with blurred pink-green transition (black arrows), next to mineralized matrix (green) [31].

stiffness, strength, and toughness [38]. In contrast to bone mineral density (BMD) as assessed by standard densitometry (DXA) *in vivo*, which is an estimate of the total amount of mineral in a scanned two-dimensional area of whole bone, bone mineral density distribution (BMDD) as assessed by quantitative backscattered electron imaging (qBEI) *ex vivo* describes the mineral content of bone matrix throughout the bone biopsy sample [38].

Human cortical and trabecular bones are formed by individual osteons and bone packets, respectively, sometimes called basic structural units (BSU). The mineral content in BSUs varies depending on the time since its deposition, which contributes to local variation in material properties of bone, which was first quantified using microradiography [39]. Subsequently, more advanced and sensitive techniques, such as qBEI, have been developed [40,41]. Micrometer-scale variations in mineral content throughout the bone tissue have been reported using a histogram curve designated BMDD [42]. The characteristic parameters of the histogram include  $C_{MEAN}$  (the average calcium content in the bone volume of investigation),  $C_{PEAK}$  (the most frequent calcium content),  $C_{WIDTH}$  (the width of the calcium content distribution),  $C_{LOW}$  (the amount of lowly mineralized bone areas), and  $C_{HIGH}$  (the amount of highly mineralized bone areas) [43].

BMDD reports mineral content in small pixels or voxels within the area of investigation [43], which is far superior to the BMD as assessed by dual-energy X-ray absorptiometry (DXA) measuring areal BMD ( $\text{g}/\text{cm}^2$ ) or quantitative computed tomography (qCT) measuring volumetric BMD ( $\text{g}/\text{cm}^3$ ). DXA and qCT estimate the total amount of mineral in a scanned whole bone including all its cavities and do not distinguish any local variations in mineral content. BMDD is reported to be essentially constant in healthy adult cancellous bone irrespective of gender, ethnicity, and biopsy site, making it a more sensitive means to detect even small changes in mineralization due to bone disease or therapeutic intervention [43]. Deviations in the histogram toward the left or right indicate hypo- or hypermineralization, respectively [38]. Individual BMDD must be compared with data obtained from similar device due to the variation in reference curves depending on the electron source used in the scanning electron microscope (SEM) [44].

In osteomalacia, the BMDD curve is broader (indicating heterogenous mineralization) and shifted to the left (indicating lower mineralization) [38]. Fig. 62.6 demonstrates back scattered electron images of a 6-month-old infant with severe nutritional rickets, demonstrating areas of poor mineralization with the BMDD curve shifted toward lower mineral content, with increased width ( $C_{WIDTH} + 55\%$ ) due to increased heterogeneity in mineralization, and markedly increased fraction of poorly mineralized matrix ( $C_{LOW} + 640\%$ ) [31]

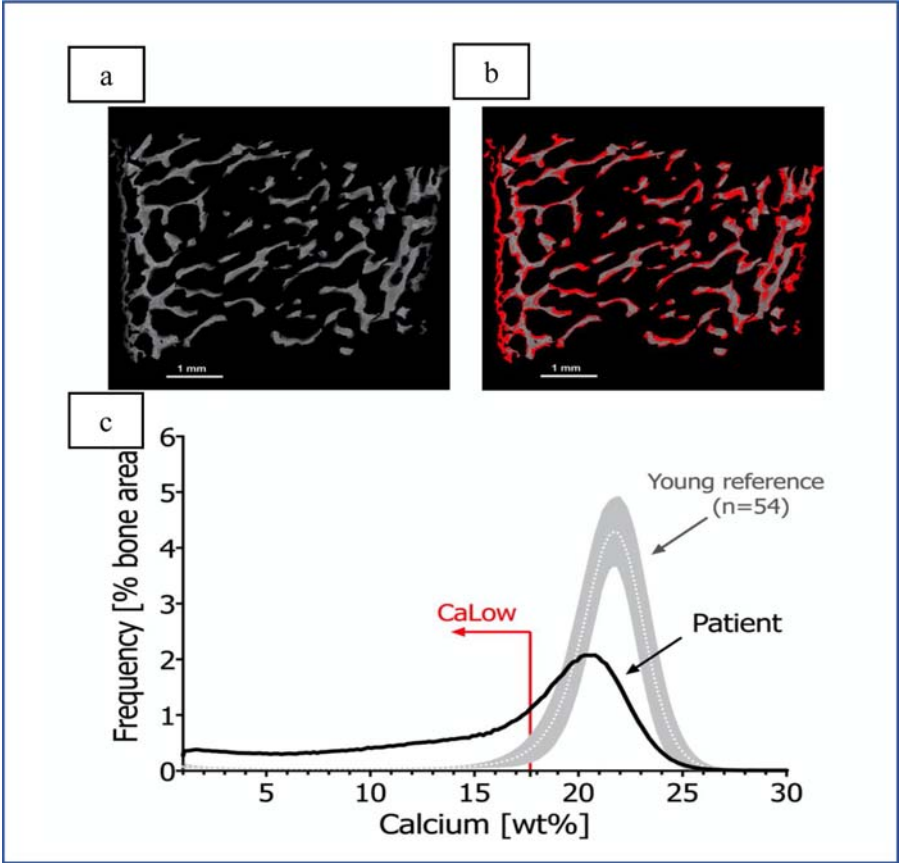
compared with the reference data from older children aged 1.5–23 years [45].

Similar to nutritional osteomalacia, individuals with other forms of rickets or osteomalacia including X-linked hypophosphataemia [46] and hypophosphatasia [47] also have a BMDD curve shifted toward lower mineralization. The BMDD curve in individuals with X-linked hypophosphatemia demonstrates lower peak and increased areas of low and heterogenous mineralization, which improves following treatment with phosphate supplements and active vitamin D [46] but does not completely reverse, and residual defects in mineralization are evident after stopping treatment [34]. In individuals with hypophosphatasia, in addition to abnormal trabecular microarchitecture, an increased number of osteoblasts compared with healthy controls or with individuals with other types of osteomalacia have been reported [47]. Following treatment with asfotase alfa (human recombinant tissue-nonspecific alkaline phosphatase) in hypophosphatasia, only minor improvements of bone microarchitecture are reported but a remarkable reduction in osteoid parameters [48]. After 2 years of asfotase alfa treatment, a significant reduction in the mineralization heterogeneity seen at baseline has been noted [48].

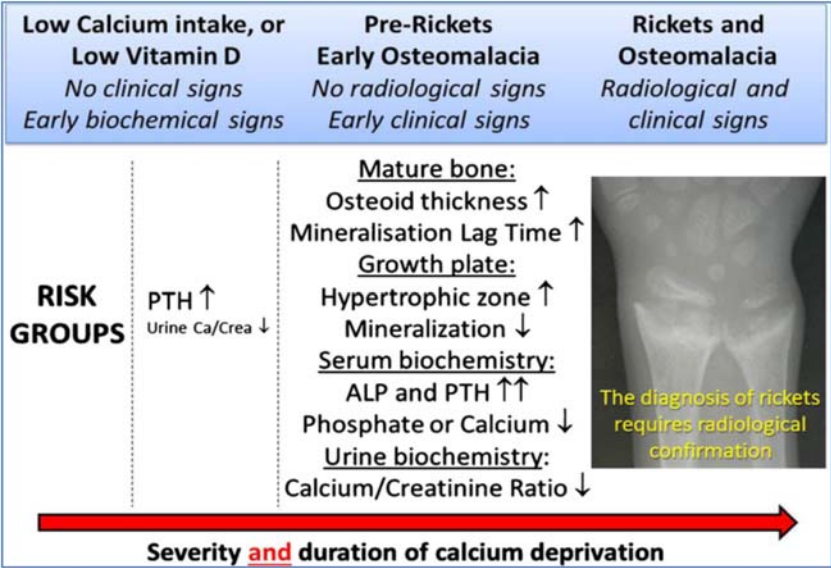
## 7. Radiological features of rickets and osteomalacia

Rickets is a radiological diagnosis (with typical biochemical and clinical features) and is evident at the metaphyses of rapidly growing bones. Signs of rickets are most evident around the wrist particularly the ulna [49], the anterior ends of the middle ribs, the proximal femur, and the distal tibia. Three stages of rickets have been described [50,51], and radiological changes are said to appear in stage two when hyperparathyroidism and hypophosphatemia secondary to increased phosphaturia are established, but features become more prominent in stage 3 [50–52] as depicted in Fig. 62.7. Although the initial stage is that of calcium deprivation, this is not manifesting clinically as hypocalcemia due to the compensatory mechanisms that come into play with secondary hyperparathyroidism [elevated parathyroid hormone (PTH)] [51]. Only those children with high calcium demand, such as infants or adolescents, are likely to present in this stage with hypocalcemic features [51,53]. In a prospective national survey of nutritional rickets in the United Kingdom and Ireland, radiological rickets was only recorded in 67% ( $n = 72/107$ ) of the children presenting with clinical and biochemical signs of rickets [54]. Similarly, another national survey determining the incidence of hypocalcemic seizures secondary to vitamin D deficiency found that of the 33%





**FIGURE 62.6** (A) and (B) illustrate backscattered electron images of the iliac bone sample of an infant with severe nutritional rickets [31]. The *dark gray* surface shows low mineral content in, *bright gray* indicates normal/high mineral content, and black is unmineralized matrix. Areas highlighted in Red (B) represent increased primary mineralization, i.e., areas mineralized below 17.68 wt% calcium, corresponding to the fifth percentile of the adult reference range (CaLow) [45]. The BMDD curve (C) is shifted toward lower mineral content compared with normative data from older children [45].



**FIGURE 62.7** Stages of calcium deprivation leading to nutritional rickets and osteomalacia [52].

( $n = 30/91$ ) who had radiographs taken, 77% (23/30) showed evidence of rickets [55]. Similar to the delayed appearance of signs of rickets on radiographs in this pathological process, complete healing of radiological rickets following treatment also takes time. On average, it takes around 3–6 months for complete radiological healing [56].

## 8. Radiographic differences between various forms of rickets

### 8.1 Radiological features of nutritional rickets

Rickets is most easily diagnosed on plain radiographs, and the classic signs include splaying, fraying, and cupping of the metaphyseal ends (Figs. 62.8 and 62.9). Pathologically, although expansion of the zone of hypertrophic chondrocytes is one of the earliest signs of rickets, it is not possible to identify this on radiographs at the onset of disease. Generally, the metaphysis and the growth plate need to be assessed separately. In the early phase of nutritional rickets, the growth plate, which appears as the radiolucent (unmineralized) gap between the mineralized metaphysis and epiphysis, widens [57]. The metaphysis, during evolution of rickets, will continuously demineralize due to PTH-induced osteoclastogenesis and thus become more and more radiolucent. Similarly, the expansion of the metaphyses or splaying becomes more evident with progressive disease. Splaying is clinically evident as swollen



**FIGURE 62.8** Wrist radiograph (anteroposterior view, left and lateral view, right) of a 3-year-old child presenting with bowing of the legs (25(OH)D 8.2 nmol/L, ALP 1003 IU/L, adjusted calcium 1.3 mmol/L, phosphate 1.2 mmol/L and PTH 136 ng/L). The radiographs demonstrate clear signs of cupping, fraying, and splaying at the metaphyses of the ulna and radius. Secondary hyperparathyroidism demonstrates a double contour in the metaphysis and diaphysis. Additionally, the diaphyseal shaft demonstrates periosteal elevation on the external cortex.



**FIGURE 62.9** Wrist radiographs of a 16-month-old presenting with hypocalcemic seizures secondary to nutritional vitamin D deficiency (25(OH)D 8.4 nmol/L, ALP 1702 IU/L, adjusted calcium 1.2 mmol/L, phosphate 1.3 mmol/L and PTH 265 ng/L). The image on the left shows classic signs of rickets of metaphyseal widening, splaying, and cupping at the ulna and radius. There is significant demineralization of the growth plate and the diaphysis. Wrist radiograph on the right shows healing of rickets (25(OH)D 164 nmol/L, ALP 343 IU/L, adjusted calcium 2.3 mmol/L, phosphate 1.58 mmol/L, and PTH 31 ng/L) following treatment with ergocalciferol and calcium. Following treatment (right), the opaque line of mineralization representing the zone of provisional calcification is visible.

joints at the wrist and ankle and as rachitic rosary at the costochondral junction [31,58]. One of the earliest radiological signs is the fading of the opaque line of zone of provisional calcification due to the decrease in mineralization of the terminally differentiated chondrocytes and cartilage. Reappearance of the line (Fig. 62.9) is also one of the earliest signs of healing rickets [49]. The loss of distinction between cartilage and metaphyseal bone along with extension of tongue like cartilage into the metaphysis (as described earlier in the growth plate histology section) results in indistinct margins or a frayed radiographic appearance. These changes are more prominent in the middle of the growth plate, which leaves a rim of calcified tissue surrounding the growth plate giving the concave or cupped appearance at the end.

A 10-point radiological severity score (RSS, or Thacher score) assessing the degree of metaphyseal fraying and cupping and the proportion of the growth plate affected at the wrists (radius and ulna) and knees (femur and tibia) is recognized as a valid outcome measure to quantify the changes of nutritional rickets [59]. Rickets severity on radiographs is reported to be correlated to serum ALP levels [59]. Some degree of subjectivity remains in reporting especially when changes of rickets are borderline or when there are alterations in the plane of the radiographs in relation to the metaphyseal–physeal junction giving a concave

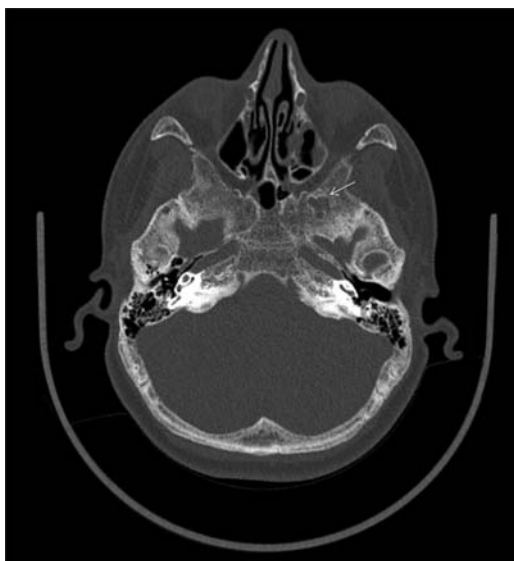
appearance to the metaphyses [51,59]. Similarly, other radiological signs of rickets such as osteopenia can be subjective and dependent on the radiographic technique. The osteoclastic resorption resulting from secondary hyperparathyroidism may be evident as subperiosteal erosion along the shaft of long bones but is a less common radiological feature of rickets compared with metaphyseal changes described before [51]. Hyperparathyroidism causes osteoclastic resorption of the Haversian canals within the cortex of the bones referred to as intracortical tunneling appearing as translucent lines on radiographs. Radiographically, this causes linear translucencies within the cortex. Excessive bone resorption in the skull vault causes the mottled texture of alternating areas of lucency and sclerosis referred to as pepper-pot skull or “salt and pepper” (Fig. 62.10) [60].

Chest radiographs are frequently done in children presenting with various health conditions and may be helpful in identifying signs of rickets and other metabolic bone conditions incidentally. Some of the common features, which lead to suspicion of bone disease or rickets, may include acute or healing rib fractures, generalized demineralization of the ribs (Fig. 62.11), or signs of rickets in the shoulder joints (Fig. 62.11). Additionally, enlarged cardiac shadow on chest radiographs

may be suggestive of cardiac failure due to dilated cardiomyopathy in infants with severe rickets (Fig. 62.12). All infants presenting with severe nutritional rickets must have their cardiac function evaluated [31,61].

## 8.2 Radiological features of osteomalacia

Following the closure of growth plates at the end of longitudinal growth, osteomalacia becomes much more difficult to spot on radiographs. Radiographic features of adolescent and adult osteomalacia can only be detected in the most severe cases. The pathognomonic feature of osteomalacia is the Looser zone fracture (pseudofracture, Milkman fracture) Fig. 62.13 [63]. Looser zone fractures are said to occur at sites of stress in the skeleton, which include the femoral neck, the medial side of the proximal femur, the pubic rami, the lateral border of the scapula, and the ribs. These must be differentiated from atypical insufficiency fractures occurring in osteoporotic bones [64] and traumatic fractures. Bones in osteomalacia are soft due to demineralization and therefore deform easily leading to varus (bowing) and less commonly valgus deformities of the lower limbs. Additional features of osteomalacia include coarse trabecular pattern at the diaphysis, biconcave vertebral deformity, protrusio acetabulae (deformity of the femoral head leading to loss of the normal acetabular margin), and triradiate appearance of the pelvis. Most importantly, clinical signs of osteomalacia includes proximal muscle weakness, with inability to rise from the floor unaided, nonspecific musculoskeletal pain, and these signs are accompanied by the typical biochemical derangement of ALP, PTH, calcium, and/or phosphate. Identifying osteomalacia based on clinical and biochemical features is possible and will help explore the hidden burden of osteomalacia enabling appropriate public health measures. Certain clinical and biochemical diagnostic criteria for identification of osteomalacia without the need for invasive bone biopsies have been proposed [65,66]. A diagnosis of osteomalacia can be made in the presence of musculoskeletal symptoms along with low serum 25(OH)D, high ALP and/or PTH, and low calcium and/or phosphate although these can be late features [65,66].

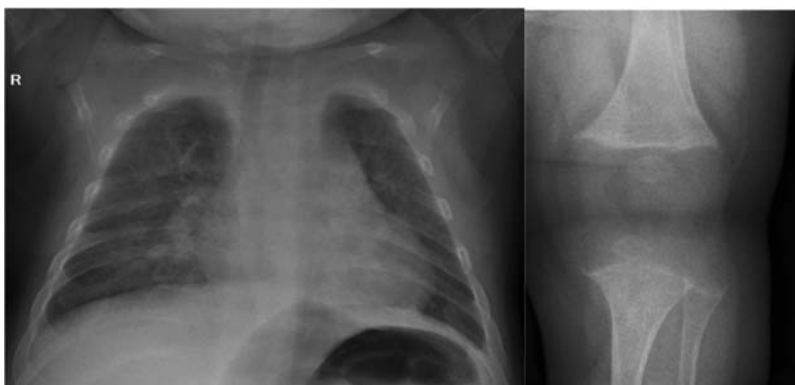


**FIGURE 62.10** Skull CT scan of an adolescent demonstrating widespread areas of alternating lucency and sclerosis with focal erosive changes in the greater wing of the left sphenoid bone (arrow). The subject had learning difficulty and restrictive eating disorder presenting with multiple and severe nutritional deficiencies including vitamin A, folate, vitamin B12, and vitamin D (25.8 nmol/L). Biochemical parameters at the time of imaging included 25(OH)D 56.2 nmol/L (on 400IU cholecalciferol supplements for 5 months), ALP 293 IU/L, adjusted calcium 2.27 mmol/L, phosphate 1.28 mmol/L, and PTH 133 ng/L. History of dietary calcium deficiency was positive and calcium supplements commenced.

## 8.3 Radiological signs of non-nutritional rickets/osteomalacia

### 8.3.1 Vitamin D–dependent rickets

To date, five distinct types of vitamin D–dependent rickets (VDDR) have been described (type 1a/b, 2a/b, 3) [67]. Apart from type 2b, all are known to be heritable with distinct genetic defects in vitamin D synthesis or degradation. The radiological features of rickets at the



**FIGURE 62.11** Incidental finding of generalized demineralization of the ribs and rachitic changes at the humerus (*left*) in a 6-month-old infant presenting with a respiratory tract infection. Further radiographs of the knee (*right*) and biochemical investigations (25(OH)D 59 nmol/L, ALP 1000 IU/L, adjusted calcium 2.46 mmol/L, phosphate 1.23 mmol/L, and PTH 24 ng/L) confirmed a diagnosis of hypophosphatemic rickets due to a very rare cause of elemental formula use [62].



**FIGURE 62.12** Chest radiograph of a 2-month-old baby demonstrating an enlarged heart due to congestive cardiac failure and dilated cardiomyopathy secondary to severe nutritional rickets (25(OH)D < 7.5 nmol/L, ALP 786 IU/L, adjusted calcium 1.6 mmol/L, phosphate 2.15 mmol/L dropped to 0.7 on initiation of intravenous calcium indicating a degree of PTH resistance, PTH 288 ng/L).

growth plate, metaphyses, and those of hyperparathyroidism along the diaphyseal shafts in VDDR are very similar to those of nutritional (vitamin D deficiency and calcium deficiency) rickets (Fig. 62.14).

### 8.3.2 Hypophosphatemic rickets/osteomalacia

Radiological feature in hypophosphatemic forms of rickets are distinctly different from those of nutritional rickets. X-linked hypophosphatemia (XLH) caused by mutations in the PHEX gene is the most common form of inherited hypophosphatemia. In XLH, the underlying pathophysiology is excess fibroblast growth factor 23 (FGF23), leading to hyperphosphaturia and hypophosphatemia. Bone mineralization in XLH is better preserved when compared with nutritional rickets due to the lack of secondary hyperparathyroidism. The



**FIGURE 62.13** Hip radiograph of a 11-year-old with Down's syndrome and global developmental delay who presented with hip pain and reduced mobility. Radiographs revealed genu valgum and bilateral Looser zone fractures (*broad arrows*) and evidence of rickets at the metaphyses (*narrow arrows*). Since he had not shown adequate response in biochemical and radiological parameters to vitamin D2 supplements (25(OH)D 52.0 nmol/L, ALP 2025 U/L, adjusted calcium 1.21 mmol/L, phosphate 2.11 mmol/L, and PTH 294 ng/L), genetic workup was initiated, which revealed VDDR1.

metaphyses are wider, and the cortices appear rather dense on X-rays (Fig. 62.15). An unusual radiological feature of XLH is the high radial trabecular vBMD (especially in treated patients) and rather low cortical vBMD measured by peripheral quantitative computed tomography (pQCT), despite histological osteomalacia in both bone compartments [34]. High-resolution peripheral quantitative computed tomography (HR-pQCT) findings in XLH patients compared with controls indicates lower trabecular numbers, greater trabecular separation, and higher trabecular network inhomogeneity with the tibia more affected than the radius [68–70]. A higher trabecular thickness in the tibia has also been reported, especially in children [71].





**FIGURE 62.14** A 11-month-old child, of consanguineous parents of Asian origin, presented with right limb pain after fall from a sofa. The initial radiograph (A) revealed no fractures but florid rickets (fraying and splaying of femoral metaphysis) was noted and a diagnosis of nutritional rickets made (25(OH)D 18.5 nmol/L, ALP 4280 IU/L, adjusted calcium 1.6 mmol/L, phosphate 0.94 mmol/L, PTH 981 ng/L). Reimaging a few weeks later due to ongoing pain revealed a fracture (B) of the right femoral shaft and evidence of ongoing rickets. Lack of expected response of biochemical parameters to treatment led to the suspicion of vitamin D–dependent rickets (VDDR) and 1-alpha hydroxylase deficiency confirmed. Radiological healing of rickets (C) and normalization of biochemical parameters was noted following treatment with alfacalcidol.



**FIGURE 62.15** Knee (A) and wrist (B) radiograph of a 3-year-old child with X-linked hypophosphatemic rickets (serum phosphate 0.7 mmol/L, ALP 777 IU/L, calcium 2.2 mmol/L, parathyroid hormone 6 ng/L) who presented with bowing of the legs, leg pain, and recurrent dental abscesses. Knee radiograph (*left*) demonstrates wide metaphyses and thickened cortex. The metaphyseal changes of rickets in the femur (A) are more prominent medially. The rachitic changes in XLH are usually more prominent at the knee than at the wrist (B). Aged 9 years, despite being on conventional therapy with oral phosphate supplements and vitamin D analogue, the child demonstrated ongoing varus deformity of the lower limbs with bilateral femoral and tibial bowing (C).

Clinically, genu varum is common in XLH, and the distal femoral and proximal tibial physes are often more affected medially than laterally. The RSS scoring system devised for nutritional rickets has also been used to evaluate disease severity and response to treatment in patients with XLH [72,73]. Despite early and lifelong treatment with phosphate supplements and active vitamin D analogs in XLH, a significant disease-related morbidity remains [73,74]. The short-term

outcomes with newer therapeutic agents such as burosumab (anti-FGF23 antibody) are promising, demonstrating radiological healing of rickets [75] (Fig. 62.16); however, long-term outcomes in the real-world remain under review. Children with XLH also typically develop dolichocephaly and craniosynostosis [76]. Radiographs in untreated, or previously treated, adults may exhibit Looser zone fractures, residual limb deformities, osteoarthritis, and enthesopathies [77].





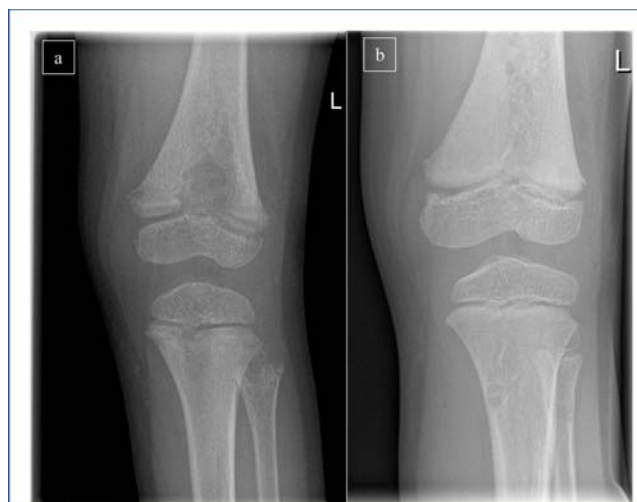
**FIGURE 62.16** Radiograph of the left knee of an 8-year-old with X-linked hypophosphatemia demonstrating metaphyseal widening and irregularity (A) on conventional therapy with oral phosphate supplements and vitamin D analogue (commenced shortly after birth). Radiological healing of rickets (B) was noted after 2 years of treatment with burosumab (anti-FGF23 antibody).

### 8.3.3 Hypophosphatasia

Hypophosphatasia (HPP) is a multisystemic bone disease caused by dominantly or recessively inherited *ALPL* gene mutations. The resulting deficiency in TNSALP reduces mineralization in growth plates, bones, and teeth and causes the hallmark biochemical feature of decreased serum ALP concentrations. Clinical variability of the condition is substantial, from severe perinatal to adult-onset disease. Generally, the earlier the presentation, the more severe the phenotype. Histological assessment of growth plates [78] and adult bone [47] confirm rickets and osteomalacia, respectively. Radiological features of the most severe, perinatal, and infantile forms of HPP are generalized hypomineralization and rickets. The typical radiological features of childhood-onset HPP include characteristic tongue-like metaphyseal radiolucency (Fig. 62.17). Other radiological features include metaphyseal fraying and widening and metadiaphyseal sclerosis. There is irregularity of the provisional zone of calcification with distal metaphyseal demineralization and a transverse subphyseal band of lucency [66]. Severe forms of the condition are associated with craniosynostosis.

## 8.4 Differential diagnosis of rickets from an imaging perspective

Above, we have described the radiological features of the classical forms of rickets. Other “nutritional” forms of rickets and osteomalacia due to deficient calcium supply or malabsorption manifest in cholestatic liver disease (where treatment is incredibly challenging [79]),

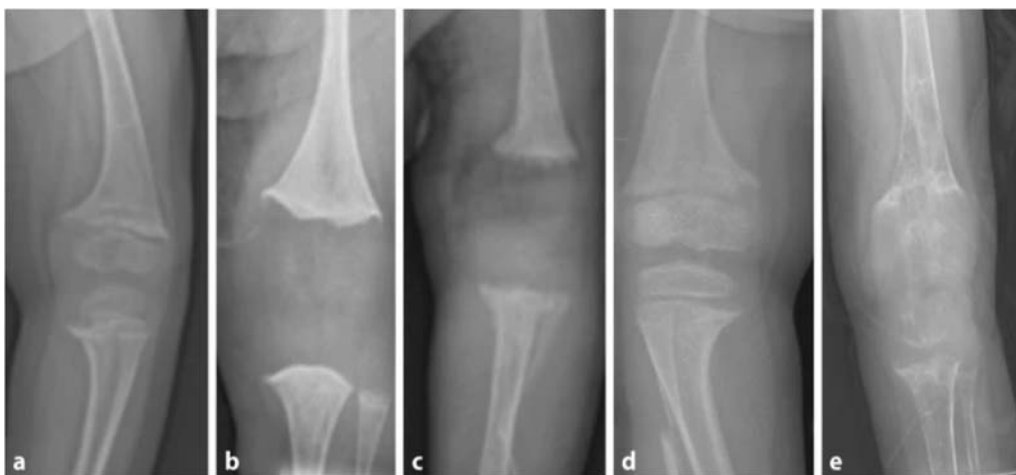


**FIGURE 62.17** Radiographs of a child with infantile hypophosphatasia pre- (A) and post- (B) treatment with asfotase alfa (recombinant, bone-targeted, human tissue-nonspecific alkaline phosphatase). The pretreatment radiograph (A) demonstrates generalized demineralization with the characteristic tongue like lucency in the femoral metaphysis. There is distal metaphyseal demineralization and a transverse subphyseal band of lucency potentially representing islands of hypertrophic chondrocytes that are migrating down the metaphysis waiting to be replaced by bone during bone remodeling.

inflammatory bowel disease, short bowel syndrome, or celiac disease. In addition, renal failure, via multiple factors including deficient 1-hydroxylation of 25(OH)D, causes rickets-like features on X-rays. Fig. 62.18 shows comparative knee X-rays in classical forms of rickets and in hepatic and renal osteodystrophy.

Rickets is often suspected in children with bowed legs and is a common reason for referral to metabolic bone specialists. The typical differentials in children under 3 years of age include physiological bowing and Blount's disease [81], a transient condition of unknown origin. Since these infants are typically early walkers (prior to 12 months) and often overweight, one theory for their bowed legs is immature biomechanical adaptability. Radiologically, there are no signs of rickets but metaphyseal beaking of the proximal medial tibial metaphysis is present (Fig. 62.19).

Severe primary growth plate disorders (skeletal dysplasias) such as achondroplasia or spondyloepimetaphyseal dysplasia may also mimic rickets radiologically although these conditions come with typical disproportionate short stature or dysmorphic features. Schmid metaphyseal chondrodysplasia, a type X collagen disorder, is associated with disproportionate short stature, bowed legs, and radiological signs reminiscent of rickets (Fig. 62.20). Rickets is also a paradoxical complication specific to autosomal recessive infantile osteopetrosis type 1 (*TCIRG1* gene mutation) caused by the inability of osteoclasts to resorb primary spongiosa, a phenomenon called osteopetrorickets [82,83].



**FIGURE 62.18** Knee X-rays with typical signs of rickets in patients with different types of rickets. (A) VDDR1, (B) renal osteodystrophy, (C) hepatic osteodystrophy, (D) XLH, (E) HPP [80].



**FIGURE 62.19** Radiograph of a 21-month-old girl with Blount's disease presenting with genu varum and waddling gait. Radiographs do not show signs of rickets but metaphyseal beaking of the proximal medial tibial metaphysis is noted.

As a general rule, when rickets is suspected, a thorough clinical and biochemical workup for hypomineralization disorders (serum ALP, PTH, 25(OH)D, calcium, phosphate, liver and renal function) is required. In the setting of normal biochemical workup primary growth plate disorders should be suspected and genetic workup considered.

## 9. Imaging choice in rickets and osteomalacia

### 9.1 Children

Classically presenting patients with nutritional rickets will display clinical signs such as leg bowing, swelling of wrists and ankles, rachitic rosary, muscle weakness, and hypocalcemic complications such as



**FIGURE 62.20** Radiograph of a 3-year-old boy presenting with bowing of legs and rickets-like changes on growth plates and metaphyses. Clinical examination and radiographs revealed rhizomelic shortening with rickets-like changes at the metaphyses. Biochemical evaluation was unremarkable (25(OH)D 93.9 nmol/L, ALP 235 IU/L, adjusted calcium 2.48 mmol/L, phosphate 1.57 mmol/L, and PTH 15 ng/L) raising the suspicion of Schmid-type metaphyseal chondrodysplasia, which was confirmed with a heterozygous *COL10A1* mutation.

seizures that should lead to rapid diagnosis purely based on clinical criteria and typical biochemistry. The minimal imaging required to confirm the diagnosis is an X-ray of wrist and knee. No further imaging techniques apart from standard X-rays are required. Patients suspected of XLH or HPP also require knee and wrist X-rays. In young children with XLH and HPP, head

circumference and skull shape need monitoring for evidence of craniosynostosis and further X-ray or CT imaging may be required for confirmation.

## 9.2 Adolescents

Adolescents with nutritional rickets/osteomalacia typically present with proximal muscle weakness and sometimes with hypocalcemic complications such as tetany. In adolescents who are close to reaching final height, growth plate activity is slow and radiological rickets (as well as the concomitant osteomalacia) can no longer be detected on wrist or knee X-rays. Although MRI may be able to detect metaphyseal abnormalities adjacent to the growth plates as demonstrated in XLH [84], MRI is neither commonly available, nor easily accessible, nor routinely indicated. Since the diagnosis can be easily made or highly suspected from clinical presentation and typical biochemistry, no further imaging is indicated. Localized pain, in particular around the thigh, should trigger further X-ray and MRI investigations to detect Looser zone fractures, which are labeled pseudofractures [85]. Confirmation of the diagnosis of suspected osteomalacia also comes with normalization of clinical symptoms and biochemistry following treatment.

## 9.3 Adults

The presentation of nutritional osteomalacia in adults can be classical with severe muscle weakness, Looser zone fractures (stress or pseudo-fractures) and typical, with typical biochemistry (elevated ALP and PTH). However, in the absence of fractures, the other signs of osteomalacia—general fatigue, malaise, muscle weakness, and pain—are nonspecific. The medical literature is surprisingly sparse on nutritional osteomalacia, even in areas where nutritional rickets is endemic in children, which may indicate that osteomalacia in adults remains largely undiagnosed [66].

Osteomalacia is regarded as a clinical diagnosis when, in fact, there are no established noninvasive diagnostic criteria. To date, the diagnosis of osteomalacia requires taking a bone biopsy. In clinical practice, typical Looser zone fractures or pseudo-fractures on X-rays, together with typical clinical and biochemical criteria, would make osteomalacia very likely. However, in the absence of fractures and validated diagnostic criteria, clinicians will be inclined to conduct a bone density (DXA) scan as osteoporosis is and remains the most common bone disease in adults. DXA, however, is unable to differentiate disorders of bone mineralization (osteomalacia) from those of reduced bone mass/structure (osteoporosis). Hence, this imaging method has no role in the specific diagnosis of osteomalacia; it will merely confirm

reduced bone mass. Similar to DXA, high-resolution pQCT will demonstrate low cortical and trabecular thickness and density but will not be able to differentiate osteomalacia from osteoporosis radiologically as seen in conditions such as tumor-induced osteomalacia [86]. Hence, even with modern imaging techniques, osteomalacia can only be suspected clinically, and imaging does not increase diagnostic certainty.

Unfortunately, these diagnostic limitations have led to integration of osteomalacia into osteoporosis management in adults, while rickets is clearly separated from osteoporosis management in children. This shortfall of medical knowledge has resulted in recommendations that all adults with low bone mass receive vitamin D and calcium preparations as a nonpharmacological baseline therapy [87]. This appears justified in the sense that older adults frequently consume low amounts of dairy products and have low sun exposure. However, such substitution is not a therapy for osteoporosis but given to prevent osteomalacia and secondary hyperparathyroidism, which can further aggravate bone loss. In that respect, it is important to recognize that giving vitamin D to people with sufficient vitamin D and dairy intake (normal habitual bone mineral supply) will not increase bone mass [88]. Clinical diagnostic criteria for nutritional osteomalacia have been proposed that do not require imaging [65,66], but these need further validation.

Overall, X-rays remain the only imaging method relevant for detection of rickets. In adolescents and adults with suspected osteomalacia with typical clinical presentation and biochemical findings, no other imaging techniques can reasonably be employed (unless for detection of suspected pseudofractures).

## 10. Conclusion

Hypomineralization disorders can result from lack of minerals (calcium and/or phosphate), mineral suppliers (calcitriol) or facilitators of mineralization such as alkaline phosphatase and nutritional rickets due to vitamin D and/or dietary calcium deficiency remain the most common cause worldwide.

Accumulation of hypertrophic chondrocytes at the growth plate, resulting from hypophosphatemia and lack of apoptosis, leads to the classic radiological features of rickets—metaphyseal cupping, fraying, and splaying—and manifests clinically with swollen ankles or wrists and rachitic rosary in children. Osteomalacia manifests clinically as bowing leg deformities in children but largely remains undiagnosed in adults, rarely presenting with Looser zone fractures. Definitive diagnosis of osteomalacia is made on histomorphometry where reduced mineralization of the osteoid is evident as increased osteoid surface, thickness, and mineralization lag on

tetracycline labeling. The BMDD curve on quantitative backscattered electron imaging is shifted to the left. Further studies are required to establish less invasive clinical and biochemical diagnostic criteria for osteomalacia.

## 11. Summary points

- Rickets is a radiological diagnosis, and children usually present with acute hypocalcemic (seizures, dilated cardiomyopathy) or hypophosphatemic (muscle weakness, bowing of legs) signs.
- Clinical presentation of osteomalacia is less nonspecific and is best diagnosed on bone biopsy with increased osteoid surface, thickness, and mineralization lag on tetracycline labeling.
- Phosphate-regulated apoptosis of hypertrophic chondrocytes is activated via the caspase-9-mediated mitochondrial pathway. Lack of apoptosis leads to accumulation of hypertrophic chondrocytes, which leads to an expansion of the hypertrophic zone as the chondrocytes, previously arranged in columns, become disarrayed.
- Radiological changes of rickets occur when hyperparathyroidism and hypophosphatemia secondary to increased phosphaturia are established.
- The changes at the growth plate—cupping, fraying, and splaying result from accumulation of hypertrophic chondrocytes due to lack of apoptosis. Secondary hyperparathyroidism results in subperiosteal erosion along the shaft of long bones, which is usually seen in calcipenic rickets secondary to calcium or vitamin D deficiency.
- Characteristic metaphyseal tongue-like lucencies are evident in hypophosphatasia, and in X-linked hypophosphatemia, widened metaphyses with dense cortices are a feature.
- DXA scan is unable to differentiate disorders of bone mineralization (osteomalacia) from those of reduced bone mass/structure (osteoporosis).

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# Vitamin D deficiency and nutritional rickets in infants and children

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## OBJECTIVES

- To present the epidemiology of nutritional rickets in children (including the metabolic bone disease of prematurity) and highlight its prevalence globally.
- To describe the various clinical presentations of vitamin D, dietary calcium and phosphorus deficiency rickets, and the influence of age and severity on these.
- To describe the characteristic perturbations in clinical chemistry associated with nutritional rickets and highlight how they differ from those associated with hypophosphatemic rickets due to renal phosphate loss.
- To outline the radiological changes associated with rickets and the use of the radiographic Rickets Severity Score in the assessment of rickets.
- To discuss the prevention and treatment of nutritional rickets, highlighting the need for global action in its prevention through possible supplementation and/or food fortification.

## Abbreviations

**1,25-(OH)<sub>2</sub>D** 1,25-Dihydroxyvitamin D  
**24,25-(OH)<sub>2</sub>D** 24,25-Dihydroxyvitamin D  
**25(OH)D** 25-Hydroxyvitamin D  
**IU** International units  
**ng/ml** Nanogram/milliliter  
**nmol/L** Nanomole/liter  
**PTH** Parathyroid hormone  
**TmP/GFR** Tubular maximum of phosphate/liter of glomerular filtrate

## Index terms

### Rickets

- pathogenesis
- diagnosis
- radiological changes
- biochemistry
- treatment
- prevention

### Vitamin D deficiency

### Dietary calcium deficiency

### 25-Hydroxyvitamin D

## 1. Introduction

Rickets is a clinical syndrome in children, resulting from failure of or delay in mineralization of the growth plate of growing bones. Of the numerous different causes, the majority can be grouped into three major categories: those that primarily result in a failure to maintain normal calcium homeostasis (calciopenic); those that primarily affect phosphate homeostasis (phosphopenic); and those that directly inhibit the mineralization process. Globally, rickets due to nutritional causes (which typically fall into the calciopenic group) remain the most frequent form of the disease. However, in a number of industrialized countries, such as the United States, the genetic forms of hypophosphatemic rickets are now probably more prevalent than nutritional causes outside the neonatal and infant periods, as a result of the fortification of foods with vitamin D and

the use of vitamin D supplements in at-risk groups. Nevertheless, the past 30 or more years have seen a resurgence of nutritional rickets primarily in dark-skinned minority communities in a number of developed countries.

## 2. Historical perspective

Although nutritional rickets is often considered to be a disease of industrialization, descriptions of rickets have been attributed to both Homer (900 BCE) and Soranus Ephesius (130 CE). Of interest is the recent report of a Neolithic skeleton of a young adult female excavated from a burial site in Scotland having deformities in keeping with those of childhood rickets [1]. The term “rickets” emerged in the hand-written “Receipt Books” of the Fairfax Family. The entry for February 25, 1632 has five remedies for “rickets in children”. The word “rickets” first appeared in print in 1634 when it figured in the Annual Bill of Mortality of the City of London for that year [2]. Perhaps the best early descriptions of rickets are provided by Daniel Whistler in 1645 and 5 years later by Francis Glisson (1650). It was described as a disease that occurred in young children, produced severe deformities, and was often fatal.

Rickets was well established in London before the industrial revolution [3]. The origin of rickets may relate to the widespread practice of wet nursing, where a wet nurse engaged in continuous breastfeeding, even nursing several children simultaneously [4]. Because the concentration of calcium in breast milk declines over the course of lactation, the calcium intake of very young infants who were wet-nursed was probably considerably less than required for adequate bone mineralization. The appearance of nutritional rickets among infants in the 17th century could have resulted from both restricted calcium intake and pollution related to burning coal in the cities [2].

With the migration of large numbers of people from rural to urban areas at the time of the industrial revolution, the disease became associated with poverty and overcrowding in the developing urban slums. In 1822, Jędrzej Sniadecki proposed that the direct action of the sun was important in the cure and prevention of rickets. Contrasting the frequency of rickets in the British Isles with the rarity of rickets in Asian countries, Theobald Palm in 1890 proposed that sun exposure could prevent rickets [5]. A number of studies in the late 19th and early 20th centuries documented the almost universal prevalence of rickets in young children in cities in northern Europe (for example, in Glasgow [6] and Vienna [7]). Harriett Chick noted that rickets had a marked winter incidence; that protection in winter could be given by diet with added cod liver oil; and that infants in the first

6 months of life were very susceptible to rickets [7]. However, with the realization of the importance of ultraviolet light in preventing nutritional rickets and the discovery and isolation of vitamin D in the first quarter of the 20th century [8] ([9] cf. Chapter 1 (Historical)), programs were introduced to prevent vitamin D deficiency.

In the United Kingdom, a number of foods were fortified with vitamin D during World War II. This led to a rapid reduction in the number of children diagnosed with rickets, but in the following years, the incidence of idiopathic hypercalcemia rose in infants, which was attributed to uncontrolled fortification of various foods (especially milk and cereals), leading to daily vitamin D intakes of 100 µg (4000 IU) or more [10,11]. There has been a resurgence of cases of rickets in the United Kingdom after reducing the levels of mandatory fortification in 1955, and lowering recommended levels of supplementation, in response to cases of hypercalcemia [12].

The prevalence of vitamin D deficiency and nutritional rickets has increased, particularly among the immigrant South Asian (Indian and Pakistani) population in the United Kingdom, and this has become a politically divisive issue, centered on the role of social welfare programs in addressing the resurgence. Rickets was framed as a disease of a transplanted minority or as an indicator of poverty among the majority [12].

In the United States, the universal fortification of milk with vitamin D at 400 IU/quart from the 1930s had almost eradicated nutritional rickets except in families who exclude milk from their diets or in infants who are breastfed for extensive periods without supplementation [13]. However, as had possibly occurred in the United Kingdom, a few cases of vitamin D toxicity have been reported as a result of the lack of monitoring of the fortification process [14]. In central Europe, rickets had been effectively prevented in infants and young children by the intermittent administration (every 3–5 months for the first 2 years of life) of high doses of vitamin D (“stoss therapie”) [15].

## 3. The epidemiology of vitamin D deficiency and nutritional rickets

Discussion and debate continue concerning the definition of vitamin D deficiency and the best methods for determining vitamin D status. Since the descriptions of the first assays to measure serum concentrations of vitamin D and/or its metabolites in the early 1970s, 25-hydroxyvitamin D (25(OH)D) has been the metabolite used to define vitamin D status, as its serum concentrations are relatively stable (half-life 2–3 weeks), are not regulated to any great extent except by substrate concentration, and reflect vitamin D intake through the diet and synthesis in the skin [16,17]. In the pediatric literature,

vitamin D deficiency has in the past been defined as that range of 25(OH)D concentrations associated with the development of rickets and osteomalacia, which have generally been accepted to occur at serum concentrations of  $<10\text{--}12\text{ ng/mL}$  or  $<25\text{--}30\text{ nmol/L}$  [18,19]. However, there is now considerable interest in the less obvious effects of vitamin D on bone mass, parathyroid hormone secretion and in the nonclassical actions of vitamin D, such as its role in immune regulation, and in the prevention of allergic and autoimmune diseases, diabetes, cardiovascular disease, and certain cancers [20,21]. This has complicated the determination of optimal vitamin D status, as there are few randomized controlled trials designed to assess optimal 25(OH)D concentrations in children taking into consideration these extraskeletal functions. A recommended dietary allowance (RDA) for vitamin D was set by the US National Academy of Medicine (formerly Institute of Medicine). Vitamin D requirements are variable across the population, and they determined the estimated average requirement (EAR) of vitamin D for the population corresponded to a 25(OH)D level of  $40\text{ nmol/L}$  with half requiring more than this and half requiring less. Based on a normal population distribution, 97.5% will have their vitamin D needs met at a serum level of  $50\text{ nmol/L}$ , and the RDA was set to achieve that level of 25(OH)D [19]. Global consensus recommendations have been developed for the prevention and management of nutritional rickets [22]. The Indian Academy of Pediatrics has developed similar recommendations [23]. These groups equate vitamin D deficiency to  $25(\text{OH})\text{D} < 30\text{ nmol/L}$ , vitamin D insufficiency to levels between  $30$  and  $50\text{ nmol/L}$ , and vitamin D sufficiency to concentrations  $>50\text{ nmol/L}$ .

Vitamin D deficiency is a prerequisite for the development of nutritional rickets in a large proportion of infants and young children. Thus the disease is typically associated with a lack of ultraviolet light, prolonged breastfeeding, and inadequate dietary vitamin D intake. As commonly ingested foods are generally deficient in vitamin D, in the absence of food fortification with vitamin D, the normal diet contributes little vitamin D [24], and adequate skin exposure to ultraviolet radiation is essential for adequate vitamin D status [25] (cf. Chapter 2—photobiology and evolution of vitamin D). Consequently, rickets occurs most frequently in infants before they are able to walk and get out-of-doors, and in children living in countries at the extremes of latitude, living in dwellings that prevent ready access to sunlight exposure or in communities in which social or religious customs prevent adequate sunlight exposure through extensive skin coverage by clothing or through the practice of *purdah*.

Vitamin D deficiency rickets is most prevalent in children under 2 years of age, with a peak incidence

between 6 and 18 months [26,27]. The disease is uncommon in infants under 3 months of age, because 25(OH)D readily crosses the placenta [28,29], thus providing the newborn infant with some protection against vitamin D deficiency if maternal vitamin D status is normal [28] (cf. Chapter 38—pregnancy and lactation). Because serum 25(OH)D has a half-life of only 2–3 weeks, serum levels fall rapidly after birth unless additional sources of vitamin D are obtained by the young infant [30]. Neonatal or congenital rickets has been described in infants born to mothers who are themselves vitamin D deficient [31–33], and neonatal hypocalcemia occurs more commonly in neonates born to vitamin D-deficient mothers [34]. The role of maternal vitamin D status during both pregnancy and lactation in predisposing infants to vitamin D deficiency has become clear [28]. Furthermore, many mothers worldwide are frankly vitamin D deficient (up to 80% in some communities [35,36]), despite some being given supplements during pregnancy [37–39]. Maternal vitamin D deficiency is associated with vitamin D deficiency rickets in their infants. In a study from the Middle East, vitamin D deficiency was identified in nearly 100% of mothers whose infants had nutritional rickets, compared with just over 50% of the mothers whose infants did not have rickets [40]. There are several reasons for this association: infants at birth are born with vitamin D stores that are dependent on the maternal vitamin D status, the breast milk of mothers who are vitamin D deficient contains negligible amounts of vitamin D, and finally the social and environmental factors that produce vitamin D deficiency in the mother are similar for the infant.

Vitamin D deficiency rickets has been noted to occur more commonly in boys than girls [41–43]; however, the mechanism for this remains unclear. It has been suggested that vitamin D deficiency rickets might be an hereditary disease, which manifests itself only under adverse environmental circumstances [42,44,45]. In a study of infants with rickets and their parents [44], urinary excretion of  $\alpha$ -amino acids was increased in one-third of the infants a long time after the rickets had healed, many of the parents had increased amino acid and phosphorus excretion, and a good correlation was found between the excretion of individual amino acids by an infant and its parents. The authors suggested that these findings indicate a genetic factor playing a role in predisposing a child to rickets; however, the mode of inheritance is unclear.

Polymorphisms of the vitamin D receptor (*VDR*) gene might play a role in predisposing infants and children to rickets. In a study of Turkish and Egyptian infants with rickets, the prevalence of the *F* allele of the *VDR* start codon was greater in children with rickets than controls [46], similar to findings in Nigerian children with dietary



calcium deficiency rickets [47] and in Asian children with nutritional rickets. Furthermore,  $1,25(\text{OH})_2\text{D}$  concentrations in Egyptian children were lower in those who were *FF* homozygotes. In the same group of children, it was suggested that the *B* allele might predispose an individual to vitamin D deficiency. However, in Indian and Mongolian studies, no relationships between rickets and various *VDR* polymorphisms were found [48,49].

*CYP2R1* is the major, but not exclusive, hepatic 25-hydroxylase responsible for the hydroxylation of the parent vitamin D to  $25(\text{OH})\text{D}$  [50]. Genetic polymorphisms of *CYP2R1* account for some of the individual variability of circulating  $25(\text{OH})\text{D}$  values in the population [51,52] and may determine vitamin D requirements in the general population. Inactivating mutations in *CYP2R1* can lead to vitamin D–deficient rickets resulting from impaired 25-hydroxylation of vitamin D [53,54] (the disease classified as vitamin D–dependent rickets, type 1B [VDDR1B, MIM 600081]).

In the early literature, breastfeeding was reported to be protective against rickets [55]. More recently, however, it has been described as a risk factor for the development of rickets [56–60]. In recent years, specifically designed breast milk substitutes have replaced natural cow's milk as the major source of nutrients for the non-breastfed infant. This alteration in feeding patterns may account for the apparent change in risk associated with breastfeeding for several reasons; firstly, breast milk substitutes are fortified with vitamin D at 400 IU/L, while natural cow's milk (unless fortified) contains little or no vitamin D [61]; secondly, the calcium:phosphorus ratio in breast milk substitutes (ratio ~2:1) is more appropriate than that in cow's milk (ratio ~1:1) for optimizing intestinal calcium absorption; and thirdly, although breast milk usually contains only small quantities of vitamin D or its metabolites (between 20 and 65 IU/L), the content is dependent on the vitamin D status of the mother [61–64]. However, there is evidence that vitamin D metabolites may cross into breast milk from the mother in sufficient quantities to maintain normal serum concentrations of  $25(\text{OH})\text{D}$  in the suckling infant if the mother receives vitamin D supplements in high doses (2000–6400 IU/day) [65–67]. A randomized controlled trial has been conducted supplementing lactating mothers with vitamin  $\text{D}_3$  at 6400 IU/day. The breastfed infants' vitamin D status was maintained through the increased breast milk vitamin D content at levels similar to those obtained by supplementing the infant directly with 400 IU/day [66], without any evidence of adverse events. Results of studies are conflicting concerning the relative concentrations of vitamin D and  $25(\text{OH})\text{D}$  in breast milk; however, what is known is that concentrations are higher in hindmilk than foremilk and correlate with maternal vitamin D status [63,68–70]. A single maternal oral dose of vitamin  $\text{D}_3$  150,000 IU was

equivalent to 5000 IU/day for 28 days in raising  $25(\text{OH})\text{D}$  values in their exclusively breastfed infants, and all infants achieved  $25(\text{OH})\text{D}$  concentrations  $>20$  ng/mL (50 nmol/L) [67]. Studies in a number of countries, including the United States, have documented poor adherence of caregivers to infant vitamin D supplementation [71–75]. Maternal supplementation with vitamin D to enrich their breast milk may be preferred to infant supplementation by some mothers, and it may avoid the potential for toxicity in the infant from dosing errors [76,77]; however, maternal supplementation to prevent vitamin D deficiency in the nursing infant cannot be recommended at present because of insufficient data on its safety or efficacy [78].

In the breastfed infant not receiving vitamin D supplements, the maintenance of an adequate vitamin D status is dependent mainly on the infant's exposure to ultraviolet light [79–81]. Specker and coworkers [79,80] have shown a marked seasonal variation in serum  $25(\text{OH})\text{D}$  concentrations in breastfed infants, which is influenced by the time spent outdoors and on the extent of skin exposed to sunlight. They have estimated that an infant in Cincinnati (latitude  $39^\circ 09' \text{N}$ ) requires to be outdoors for either 20 minutes a week in a diaper only or for 2 hours a week fully clothed but without a hat to maintain circulating concentrations of  $25(\text{OH})\text{D}$  above 11 ng/mL (27.5 nmol/L), which might by today's criteria not be considered optimal levels [79]. In a study conducted in Delhi ( $28.7^\circ \text{N}$ ), it was calculated that to achieve  $25(\text{OH})\text{D}$  of  $>20$  ng/mL (50 nmol/L) at 6 months of age, breastfed infants would be required to expose 40% of the body to 30 min weekly of sunshine (between 10 a.m. and 3 p.m.) for at least 16 weeks [82].

Seasonal variations in serum  $25(\text{OH})\text{D}$  concentrations have also been documented in a number of countries in older children and adults [83–85], and these variations appear to correlate with the amount of ultraviolet light reaching the earth [25]. These observations highlight the importance of the photo-biosynthesis of vitamin  $\text{D}_3$  in the skin to prevent vitamin D deficiency and thus rickets in many populations in the world [86]. In a number of countries such as Turkey [87], Saudi Arabia [58], India [88], Bangladesh [89], Iran [26], Kuwait [90], Kenya [91], Nigeria [92], Egypt [93], Ethiopia [94], and in others in the tropics and subtropics [95–99], rickets remains a problem despite generally good daily hours of sunshine. Several factors contribute to the persistence of the problem in these areas: overcrowding and poverty, atmospheric pollution [100], purdah, lack of access to sunlight, a lack of vitamin D–fortified foods or regular vitamin D supplements, and diets low in calcium and high in inhibitors of calcium absorption [101–106].

Despite its near eradication, the prevalence of nutritional rickets is increasing in high-income countries,



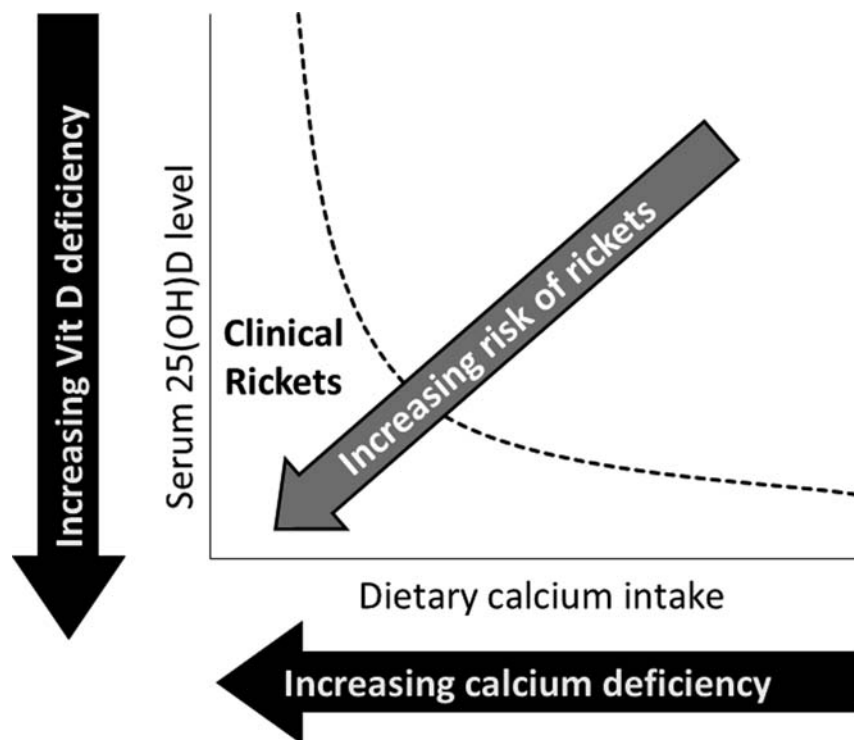
largely driven by an increased influx of immigrant and refugee children from parts of the world with a high prevalence of nutritional rickets [107–114]. Nutritional rickets is most frequent in children from the Middle East, Africa, and South Asia, even though many of these countries are located in the tropics with abundant sunshine [102–106]. Thus, there is now good evidence that nutritional rickets is a global public health problem, and not just the preserve of low- and middle-income countries (LMICs).

In the United States, despite the almost complete eradication of vitamin D deficiency among Caucasian children, several studies have highlighted the resurgence of the problem in specific groups [13,56,57,109,115–120], namely vegans and children on macrobiotic diets, children who are breastfed for prolonged periods, and African American children [120,121]. It is suggested that the combination of decreased vitamin D<sub>3</sub> formation in the dark skin, extensive skin coverage by clothing, low dietary vitamin D intakes because of the lack of intake of dairy products, and the generally low dietary calcium intakes associated with vegetarian diets contribute to an increased risk for nutritional rickets in these groups.

A similar pattern has also been documented in darker skinned immigrant populations in a number of European countries [108,110,122–125], North America [109,126], and Australia [107,127] and New Zealand

[128–130]. Since the initial descriptions of the resurgence of rickets in the United Kingdom in the early 1960s, numerous studies have been undertaken to determine why Asians are predisposed to the problem when other immigrants such as Afro-Caribbeans are thought to be less at risk [131]. Among the hypotheses put forward are simple vitamin D deficiency due to the dark skin and lack of skin surface exposed to sunlight [132,133], low calcium diets associated with vegetarianism [134], and impaired intestinal calcium absorption associated with high phytate diets [135]. A unifying hypothesis, proposed by Clements [136], suggests that in a situation of relative vitamin D insufficiency, the low dietary calcium and high phytate content of the typical Asian vegetarian diet leads to mild secondary hyperparathyroidism and a resultant increase in the catabolism of vitamin D. The progressive decline in vitamin D status culminates in the development of rickets.

The risk of nutritional rickets is a function of both vitamin D status and calcium intake [137]. Although rickets can result from either vitamin D deficiency or calcium deficiency, more commonly these two conditions interact to increase the risk of developing rickets (Fig. 63.1). The interaction of vitamin D and calcium to maintain bone mineralization is such that an adequate intake of either one will generally compensate for sub-optimal intake of the other. However, when both



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**FIGURE 63.1** A graphic representation of the synergistic effects of low dietary calcium and vitamin D status on the development of nutritional rickets. *Reproduced with permission from Dr. T. D. Thacher.*

calcium intake and vitamin D status are low, rickets can develop [138]. Vitamin D requirements are greater when dietary calcium intakes are low [137,139].

#### 4. Clinical presentation

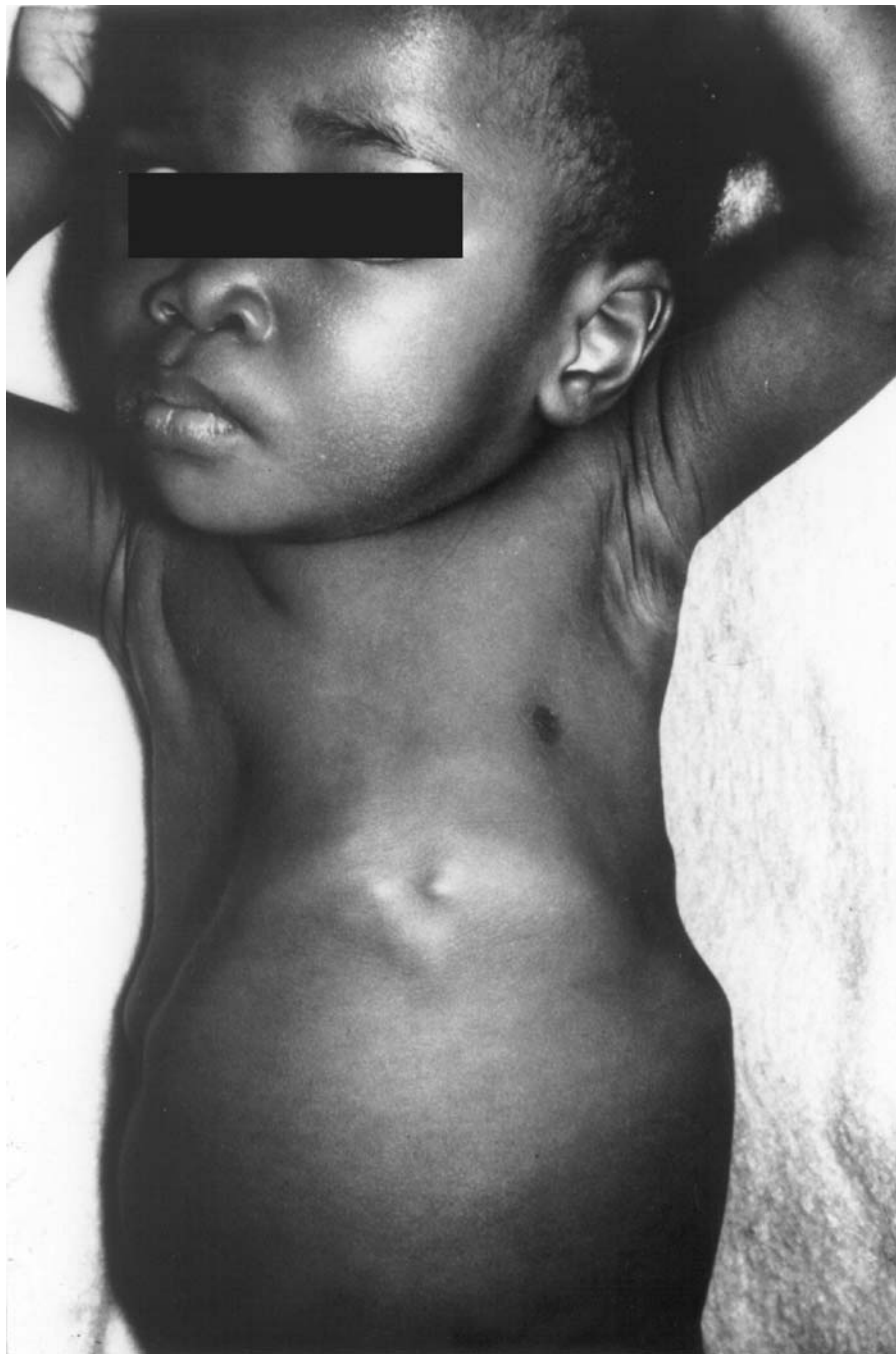
Most clinical signs in children with rickets result from the effects of vitamin D deficiency on the mineralization process at the growth plate or on calcium homeostasis. Fraser and coworkers [43] described three stages in the progression of vitamin D deficiency. Stage I is characterized by hypocalcemia with clinical signs related to hypocalcemia. In stage II, the clinical features of impaired bone mineralization appear, and in stage III, signs of both hypocalcemia and severe rickets are present. This division of the progression of vitamin D deficiency rickets is conceptually useful, but there is considerable clinical overlap between the various stages.

The early clinical manifestations of vitamin D deficiency (stage I) related to hypocalcemia are seen most commonly in young infants (less than 6 months of age). They may present with convulsions [140,141], apneic episodes [142], tetany, cardiac failure, or even cardiac arrest [143], with no clinical signs of rickets. Children with symptomatic hypocalcemia are often not recognized to have vitamin D deficiency [144] despite even having radiologic evidence of rickets. In a population-based study of children aged 0–5 years in Olmsted County, Minnesota, the incidence of hypocalcemia with a potentially life-threatening complication was 6.1 per 100,000 person-years [144]. 25(OH)D was measured in only 3 of 16 children with symptomatic hypocalcemia [145]. Pseudotumor cerebri [146] and cataracts, probably due to hypocalcaemia, have also been reported in a young infant with rickets [147]. It has been suggested that symptomatic hypocalcemia in infants with vitamin D deficiency might be precipitated by an acute illness [148], during which there is a release of intracellular phosphate [149]. Why young infants are particularly predisposed to presenting in stage I is not clear, but it may relate to a delayed response of the parathyroid glands to hypocalcemia. In addition, these infants tend to have higher serum phosphate levels [150,151]; thus, the classical clinical and radiological features of rickets may not be evident during early infancy and at this stage of the disease.

Most children with vitamin D deficiency pass through the early phase of hypocalcemia asymptotically, presenting later with clinical features of rickets. Typically the infant or young child presents with a delay in motor milestones, hypotonia, irritability, and progressive deformities of the long bones. The deformities are most noticeable at the distal forearm with enlargement of the wrist and possible deformities of the distal radius

and ulna, and in the legs with progressive lateral bowing of the femur and tibia. The sites and types of deformity are dependent on the age of the child and the weight-bearing patterns in the limbs. Thus in the small infant, deformities of the forearms and anterior bowing of the distal tibias are more common, while in the toddler who has started to walk, an exaggeration of the normal physiological bowing of the legs (genu varum) is characteristic. In the older child, valgus deformities of the legs or a windswept deformity (valgus deformity of one leg and varus deformity of the other) may be apparent. The characteristic feature in the ribs is enlargement of the costochondral junctions, leading to visible beading along the anterolateral aspects of the chest (the rachitic rosary). Rachitic rosary was the most frequent clinical sign reported in children aged 6–18 months with rickets in Pakistan [152]. In the infant or young child with severe rickets, the muscular pull of the diaphragmatic attachments to the softened lower ribs results in the development of an Harrison sulcus (Fig. 63.2). The negative intrapleural pressure associated with breathing may result in narrowing of the lateral diameter of the chest (the violin case deformity) with consequent severe respiratory embarrassment. Increased sweating has also been described in young infants and probably relates to the increased work of breathing due to the decreased compliance associated with the excessively malleable ribs. In premature infants with rickets (which may also be due to dietary phosphorus deficiency), rib or forearm fractures may be the first clinical sign to draw attention to the problem [153].

Skull abnormalities associated with nutritional rickets include a delayed closure of the fontanelles, parietal and frontal bossing (hot-cross bun appearance), craniotabes [154], and craniosynostosis. Frontal bossing and widened fontanelles have been described as common features of breastfed infants with rickets in Pakistan [155]. Craniotabes is often considered to be highly suggestive of rickets if other causes such as hydrocephalus and osteogenesis imperfecta have been excluded. A number of studies have suggested craniotabes might be a normal finding in healthy young infants [156–158], although other studies in neonates have found an association with maternal vitamin D deficiency [159]. A large study in Japan showed the prevalence of craniotabes was affected by season of birth, and at 1 month of age, infants with craniotabes had a higher prevalence of elevated alkaline phosphatase concentrations, hyperparathyroidism, and low 25(OH)D levels [154]. Craniosynostosis, involving the coronal or multiple sutures, has been described in approximately 25% of children, who were followed up after having suffered from vitamin D deficiency rickets [145,160]. The development of craniosynostosis appears to be related to the degree of severity of the rickets and thus to the severity



**FIGURE 63.2** An infant with vitamin D deficiency rickets, presenting with respiratory distress. The child shows the characteristic deformities of the chest associated with severe rickets. The lateral diameter of the chest is reduced, and bilateral Harrison's sulci are present. The abdomen has a protuberant appearance. *Reproduced with permission from Ref. [240].*

of the mineralization defect, and inversely to the age of onset of the rickets.

A delay in tooth eruption is a feature of rickets in the young child, and enamel hypoplasia of teeth may occur if rickets develops prior to the completion of enamel deposition [161]. The latter has been reported in the primary dentition of infants born to mothers who are vitamin D deficient [162], and is seen in the secondary

dentition of children who have suffered from rickets during early childhood [161]. A number of studies have also found inverse associations between childhood vitamin D status and dental caries in childhood [163].

Hypotonia, decreased activity, and a protuberant abdomen are characteristic features of advanced vitamin D-deficient rickets in the infant and young child. These signs are probably analogous to the proximal muscle

weakness described in vitamin D–deficient adolescents and adults [164]. In this situation, deep tendon reflexes are retained and may be brisk. The pathogenesis of the myopathy is thought to be due primarily to vitamin D deficiency, rather than hypophosphatemia [165] (cf. Chapter 33—muscle).

Children with rickets typically have impaired growth and delayed motor milestones [130], which may be related to bone pain and muscle weakness. Although leg deformities can contribute to stunted height, restricted growth plate mineralization is probably a more important factor in limiting growth velocity in nutritional rickets [166]. Among the anthropometric parameters, height-for-age z-score is most severely reduced [167]. Nutritional rickets presents with general weakness, leg pain with walking or at rest, frequent falls, and inability to walk after infancy [167]. Five clinical features that were most predictive of active calcium deprivation rickets in Nigerian children were age <5 years, height-for-age less than 2 SD below the mean, leg pain during walking, wrist enlargement, and costochondral enlargement [168].

A child may have bone deformities characteristic of rickets, but radiographs do not show active rickets and biochemical abnormalities. Such children may have had active rickets earlier that has healed spontaneously, if the child has been able to catch up with the demands for calcium and vitamin D. Bone deformities were found in 3.3% of children clinically screened for rickets in The Gambia, but only 9% of those with deformity had active rickets on X-ray [169]. Among Nigerian children over age 18 months presenting with bone deformities, 38% had radiographically active rickets [168]. Radiographic confirmation is essential to determine if rickets is active.

Dilated cardiomyopathy and cardiac failure [170,171] have also been described in young infants with vitamin D deficiency and have been described as one of most serious complications of vitamin D deficiency [172]. Infants can present either with rickets or dilated cardiomyopathy as the initial diagnosis [171,173]. If a holistic approach to the investigations and management of these children is not adopted, then either of these two pathologies can be missed when presenting concurrently. In one series of the 18 infants diagnosed with vitamin D deficiency and cardiomyopathy, eight were ventilated and three died, indicating the severity of this complication in young infants [174]. The mechanism is thought to be due to the effect of hypocalcemia on cardiac muscle function, rather than a direct effect of hypovitaminosis D [175]. These abnormalities returned to normal after treating the rickets [175].

Infants and young children with rickets are prone to an increased number and severity of infections [176,177]. The prevalence of nutritional rickets among children under age 5 years in Uganda with severe

pneumonia was 9.5% [178]. Although the increase in respiratory infections may be explained on the thoracic cage abnormalities (softening of the ribs, the enlarged costochondral junctions, and the decreased thoracic movement due to muscle weakness), the now well-documented role of 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) in modulating immune function [179–181] may contribute to the observed increase in infections. Impaired phagocytosis [182] and neutrophil motility [183] have been described in children with vitamin D deficiency rickets, and the lack of production of cathelicidin following the activation of Toll-like receptors may play a role in predisposing vitamin D–deficient subjects to *Mycobacterium tuberculosis* infection [184].

A possibly associated abnormality is anemia, thrombocytopenia, leukocytosis, myelocytosis, erythroblastosis, myelofibrosis [185], myeloid metaplasia, and hepatosplenomegaly (von Jaksch–Luzet syndrome) [186], which has been described in infants with rickets [187]. Although the exact pathogenetic mechanisms for this syndrome are unclear, vitamin D deficiency has been implicated based on the clinical observation that vitamin D therapy cures the condition and on experimental evidence showing that  $1,25(\text{OH})_2\text{D}$  has antiproliferative activity on myeloid leukemia cell lines [188].

## 5. Biochemical abnormalities

The hallmark of vitamin D deficiency is a low circulating level of  $25(\text{OH})\text{D}$ . In healthy children and adults, a range of approximately 12.5–50 ng/mL (30–125 nmol/L) has been found in the majority of studies [84,189–191] conducted in communities in which vitamin D–deficient rickets is uncommon. However, the normal range is dependent not only on the vitamin D and calcium contents of the diet and on the ultraviolet light exposure of the skin, but also on the definition of vitamin D deficiency. In a number of studies, a marked seasonal variation in levels has been recorded [83,84,192], reflecting in part the seasonal changes in the amount of ultraviolet light reaching the earth. During the winter months, at latitudes above 40°N or S, insufficient UV radiation reaches the earth to allow any cutaneous synthesis of vitamin D [25,193]. In countries at high latitude where foods are not vitamin D fortified, serum  $25(\text{OH})\text{D}$  concentrations in some “normal” children may be in the range documented in symptomatic children with vitamin D deficiency [83,194]. Thus the development of symptoms depends on the duration and severity of low  $25(\text{OH})\text{D}$  concentrations and on the ability of the kidney to achieve adequate  $1,25(\text{OH})_2\text{D}$  concentrations in the face of decreased substrate for the gastrointestinal tract to maintain calcium absorption at a level appropriate to meet the



demands of the growing child. In children with 25(OH)D concentrations within the normal reference range, there is no correlation between serum 25(OH)D and 1,25(OH)<sub>2</sub>D concentrations. However, once 25(OH)D levels fall below ~12 ng/mL (30 nmol/L) 1,25(OH)<sub>2</sub>D concentrations correlate with those of 25(OH)D [195–197]. In the majority of studies in which 25(OH)D values have been measured in children with vitamin D deficiency rickets, concentrations have been found to be less than 4–5 ng/mL (10–12.5 nmol/L) in most patients [40,194,198], although other researchers have found higher values [199–201].

The classical biochemical changes in vitamin D-deficient children who have radiological changes of rickets are a combination of hypocalcemia, hypophosphatemia, and elevated alkaline phosphatase and parathyroid hormone (PTH) concentrations. Over 50 years ago, Fraser and coworkers [39] elegantly described the biochemical progression in children with rickets as vitamin D deficiency became more severe and prolonged. In the early phase of vitamin D deficiency before the development of radiological signs (stage I), hypocalcemia may be the only biochemical abnormality [43]. Acute illness may precipitate hypocalcemia in the vitamin D-depleted infant through the sudden increase in serum phosphorus concentrations [149]. The biochemical picture is such that stage I rickets may be confused with that of hypoparathyroidism or pseudohypoparathyroidism [202], as hypocalcemia, hyperphosphatemia, and normal alkaline phosphatase levels may also be found [203]. As vitamin D deficiency progresses, secondary hyperparathyroidism in response to the hypocalcemia induces a partial correction of the low serum calcium concentration, which may return to levels within the normal range, and increases phosphate excretion by the kidney resulting in hypophosphatemia (stage II) [204]. At this stage, serum alkaline phosphatase concentrations are usually elevated, and other renal manifestations of secondary hyperparathyroidism, such as increased cyclic AMP excretion, generalized aminoaciduria, impaired acid excretion, and decreased urinary calcium excretion, are found [205]. In stage III of the disease, the radiological features are more severe, hypocalcemia once again becomes apparent, and alkaline phosphatase concentrations rise further [43]. Evidence of end-organ resistance to PTH has been found in young children with both mild and more severe radiological rickets [206,207]. In a study by Kruse [206], children with mild rickets remained normophosphatemic and had normal renal handling of phosphate (TmP/GFR) despite elevated PTH concentrations and increased urinary cyclic AMP excretion. Similar indirect evidence of PTH resistance (hypocalcemia, normophosphatemia, and a decrease in the renal phosphate excretion index) was noted by Taitz [207] in infants with more severe

radiologic rickets. Resistance to PTH has also been described in hypocalcemic adolescents with mild rickets [208]. Usually, however, as the severity of the rickets increases (stages II and III), PTH values rise further, and renal hyporesponsiveness is overcome [206]. Thus, hypophosphatemia and a decrease in TmP/GFR become hallmarks of the disease.

## 6. Biomarkers of active rickets

Urine calcium to creatinine ratios were found to be very low in rachitic Nigerian children and lower than in control children without rickets [167]. However, neither the urine calcium to creatinine ratio nor urine phosphorus was related to serum 25(OH)D in US children without nutritional rickets, and the investigators concluded that urinary calcium and phosphorus were not useful screening tests for vitamin D deficiency [209].

Markers of bone turnover are typically elevated in nutritional rickets in response to the development of secondary hyperparathyroidism. Urinary hydroxyproline excretion may be within the normal range in stage I rickets, but is elevated in patients with radiologic rickets [206], and an increase in serum concentrations of bone resorption markers has been reported in children with untreated rickets [210,211] and in those with asymptomatic vitamin D deficiency [212]. Similarly, serum alkaline phosphatase values may be normal in stage I of vitamin D deficiency, but rise with the degree of severity of the radiologic changes. On treatment, bone turnover markers (especially those of bone resorption) rise in the first 2–3 weeks and then fall progressively to normal values over a period of 4–6 weeks [211]. Of all the readily available biochemical tests that might be deranged in nutritional rickets, alkaline phosphatase has been used most frequently as a screening test. However, although it is elevated in nearly all children with radiological changes, it lacks specificity, and normal values vary with age [213]. Serum alkaline phosphatase levels are generally related to the radiological severity of rickets [95,214]. Although cholestatic liver disease is associated with vitamin D deficiency and elevated liver alkaline phosphatase, bone specific alkaline phosphatase [215] is generally not required to identify the source of elevated alkaline phosphatase, if radiological changes confirm the diagnosis of rickets and values improve with treatment. Elevated alkaline phosphatase effectively discriminated between Nigerian children with and without nutritional rickets [216]. Alkaline phosphatase is a low-cost biochemical test that could be used to screen for nutritional rickets, but cutoff values require validation in other populations, and laboratory values need to be standardized for widespread population studies [216].



In a small study of rachitic subjects, the authors suggested that the measurement of deoxypyridinoline in a first morning void urine sample might be a useful indicator of rickets, values being significantly higher in patients than in age matched controls [217]. In a case–control study of urinary biomarkers in children with and without nutritional rickets in China, the combination of urinary phosphate and sebacic acid had high sensitivity (94.0%) and specificity (71.2%) for nutritional rickets [218].

Osteocalcin is a noncollagenous bone matrix protein that binds to hydroxyapatite and is secreted by osteoblasts during mineralization [219]. Serum concentrations are higher in children than adults and peak during the pubertal growth spurt [220]. In the few children with untreated vitamin D deficiency rickets, in whom serum osteocalcin concentrations have been measured, values have been reported to be low [211] or normal [221], and may rise rapidly on therapy to supranormal concentrations [222]. One Nigerian study [210] of 12 rachitic children found slightly elevated serum osteocalcin concentrations compared with values in age-matched controls, while another found levels to be similar or slightly lower in rachitic children than controls [223]. The children in both these studies had low dietary calcium intakes.

In patients with vitamin D deficiency, serum  $1,25(\text{OH})_2\text{D}$  concentrations have been reported to be low, normal, or even elevated [195,198,201,206,224,225], while  $24,25(\text{OH})_2\text{D}$  values are low or undetectable [195,198,201,224,226]. Kruse [206] found that  $1,25(\text{OH})_2\text{D}$  values were higher in children with stage II rickets than in those with either stage I or stage III rickets. The finding of normal or elevated levels of  $1,25(\text{OH})_2\text{D}$  in vitamin D deficiency rickets has led some researchers to conclude that other vitamin D metabolites, such as  $24,25(\text{OH})_2\text{D}$ , are necessary for the maintenance of normal calcium homeostasis [225,227,228], but the evidence for this is not convincing. Others have suggested that although concentrations are within the normal range, they are inappropriately low for the degrees of hypocalcemia and hyperparathyroidism [206,224]. As discussed later, the latter hypothesis is more likely.

One indicator of relative or functional vitamin D deficiency that has been observed in nutritional rickets is a rapid rise in  $1,25(\text{OH})_2\text{D}$  levels in response to vitamin D administration [229]. This has been interpreted as a response to vitamin D administration in a situation of a relative lack of substrate  $25(\text{OH})\text{D}$  in the presence of PTH stimulation of renal  $1\alpha$ -hydroxylase.

Circulating concentrations of IGF-1 in children with nutritional rickets were positively correlated with  $25(\text{OH})\text{D}$  and increased significantly after treatment of rickets with vitamin D [166]. Growth velocity after

treatment of active rickets was related to the increase in IGF-1 and  $25(\text{OH})\text{D}$  levels, suggesting that accelerated linear growth after treatment of nutritional rickets may be mediated by IGF-1 secretion.

The possible pathophysiological progression of vitamin D deficiency rickets in children may be described as follows [206,230]. As the child becomes progressively vitamin D depleted, a stage is reached when the serum  $25(\text{OH})\text{D}$  concentration falls below that required to maintain a serum  $1,25(\text{OH})_2\text{D}$  level necessary to ensure the required intestinal calcium absorption for normal calcium homeostasis. The resultant hypocalcemia (stage I rickets) leads to secondary hyperparathyroidism, which, through the stimulation of  $1\alpha$ -hydroxylase (CYP27B1), increases  $1,25(\text{OH})_2\text{D}$  production despite falling  $25(\text{OH})\text{D}$  concentrations. In concert with PTH,  $1,25(\text{OH})_2\text{D}$  increases bone resorption and intestinal calcium absorption, thus returning serum calcium concentrations toward normal (stage II rickets). The presence of hypophosphatemia at this stage is probably responsible for the mineralization defect and the development of radiologic rickets.

It is during stage II that serum  $1,25(\text{OH})_2\text{D}$  concentrations may be elevated [206]. A possible explanation for the failure of the elevated  $1,25(\text{OH})_2\text{D}$  levels to reduce the hyperparathyroidism and heal the bone disease at this stage is that the concentrations are not high enough to meet the increased calcium requirements associated with the generalized mineralization defect and increased bone turnover. Support for this hypothesis comes from data, which show that  $1,25(\text{OH})_2\text{D}$  concentrations rise to considerably higher levels (3–5 times normal) during the healing process even when only small doses of vitamin D are provided [195,206] and that intestinal calcium absorption may reach ~80% of dietary calcium intake during this phase [195,231]. The hyperparathyroidism can lead to a further decrease in  $25(\text{OH})\text{D}$  concentrations through both increased conversion to  $1,25(\text{OH})_2\text{D}$  by CYP27B1 and increased degradation by  $24$ -hydroxylase (CYP24A1), induced by  $1,25(\text{OH})_2\text{D}$  [232,233].

As  $25(\text{OH})\text{D}$  concentrations fall further,  $1,25(\text{OH})_2\text{D}$  levels once again fall, despite persistent hyperparathyroidism, because of the lack of substrate. However, this does not typically occur in adults unless the  $25(\text{OH})\text{D}$  concentrations fall below 10 nmol/L (4 ng/mL) [234]. Hypocalcemia again becomes apparent as intestinal calcium absorption falls and calcium mobilization from bone decreases due to the extent of unmineralized osteoid covering bone surfaces and the lack of  $1,25(\text{OH})_2\text{D}$ , which has a permissive action on bone resorption by PTH [235,236]. The combination of both hypocalcemia and hypophosphatemia increases the severity of the bone disease (stage III).

## 7. Radiologic changes

The typical radiologic changes associated with nutritional rickets have been well described and are discussed in Chapter 54—radiology. Radiographic features of rickets reflect disordered mineralization and ossification of the growth plate [237]. Stage I rickets characteristically shows few radiologic signs, although diffuse demineralization caused by secondary hyperparathyroidism might precede the changes at the growth plate [237]. The changes of rickets are best visualized at the growth plate of rapidly growing bones. Thus, in the upper limbs, the distal ulna may show best the early signs of impaired mineralization [238]. In the older child, the metaphyses round the knees become more useful. Other radiologic findings include fractures, periosteal new bone, and bowing deformities [237].

The early signs of rickets include widening of the physal plates and a loss of definition of the provisional zone of calcification at the metaphyses [239]. As the disease progresses, the disorganization of the growth plate becomes more apparent with cupping, splaying, spur formation, and stippling [149,240] (Fig. 63.3). The appearance of epiphyses may be delayed, or they appear small, osteopenic, and ill-defined.

As noted before, not all children with rachitic-like bone deformities have active rickets at the growth plates. In Nigerian children, an age below 5 years, height-for-age Z-score less than 2, leg pain during

walking, wrist enlargement, and costochondral enlargement were independently predictive of active rickets on radiographs [168]. Any three of these clinical features accurately identified 87% of children with active rickets. Among children in Pakistan, 2–36 months old, with clinical signs of rickets and elevated alkaline phosphatase, radiological findings suggestive of rickets were found in 54% [152].

A widely used radiographic severity score (RSS) has proven useful to quantify the severity of radiographic changes of rickets, based on wrist and knee radiographs [169,214,241–243]. The score ranges from 0 to 10, where 10 represents the most severe rickets. The RSS correlates well with serum alkaline phosphatase values. The RSS has been independently validated in low-income and high-income countries, and it is particularly useful for clinical trials involving children with rickets to numerically measure the severity of rickets [46,238,244]. The RSS was independently validated in children with rickets in India, and the distal ulna was the last to heal in most cases [238]. These investigators concluded that the distal ulna could be used for diagnosis and follow-up in nutritional rickets, except in severe cases, where the distal femur was a better radiological indicator. They derived a linear regression formula to predict the expected time for radiological resolution, based on RSS. Along with the RSS, a radiographic global impression of change (RGI-C) score has been used to assess improvement in genetic forms of rickets, such as



**FIGURE 63.3** The radiographic features of vitamin D deficiency rickets at the wrist. *Left panel:* untreated vitamin D deficiency showing underdevelopment of the epiphyses, widening of the physal plates, splaying and irregularity of the metaphyses, and loss of the provisional zone of calcification. The shafts of the radius and ulna show coarsening of the trabecular pattern and loss of the normal cortical definition. *Middle panel:* response after 3 months of vitamin D therapy. The metaphyses show clear signs of healing with dense bands of calcification at the distal ends of the metaphyses, narrowing of the physal plates, and more clearly defined epiphyses. The trabecular pattern still appears coarse but shows improvement. *Right panel:* six months after starting vitamin D therapy. The radiographic changes of rickets have disappeared. The epiphyses, physal plates, metaphyses, and trabecular structure are normal. *Reproduced with permission from Ref. [240].*

hypophosphatasia and X-linked hypophosphatemic rickets [245,246]. The RGI-C could also prove applicable to assess healing in studies of nutritional rickets. An artificial intelligence object detection model has reasonable sensitivity and specificity to identify rickets on pediatric wrist radiographs [247].

The shafts of the long bones show features of both hyperparathyroidism and osteomalacia. Osteopenia is a characteristic feature, which in the so-called “atrophic” form of the disease may be very severe [95]. The cortices become thin and may show periosteal new bone formation, although this is more frequently seen during healing. The trabecular pattern is reduced and appears coarse. Deformities of the shafts of the long bones are typically present, and in severe rickets, pathological fractures and Looser zones may be noted. In vitamin D-deficient rickets, features of hyperparathyroidism, such as subperiosteal erosions, are uncommon. However, loss of the lamina dura around the teeth is frequently seen.

Enlargement and splaying of the costochondral junctions on the lateral radiographs of the chest have been used as a sign of rickets; however, in one study, mild changes were found to be unreliable as their presence did not correlate with serum 25(OH)D concentrations or with other features of rickets at the distal radius and ulna [248].

Rickets during adolescence may be difficult to detect using the conventional radiographic sites of the wrist and knees as the epiphyseal plates narrow and epiphyses fuse. A radiograph of the pelvis may be useful in this situation as the secondary iliac and ischial ossification centers (apophyses) may be abnormally wide [249]. These centers appear at puberty and normally unite with the rest of the bone between the 15th and 25th years of age.

The earliest sign of healing of rickets is the appearance of broadened bands of increased density corresponding to the zone of provisional calcification in the distal metaphysis, often apparent within 6 weeks of treatment [250] (Fig. 63.3). The demarcation of the broad bands on the diaphyseal side of the shaft may be poorly defined [239]. Healing in more severe cases of rickets may first appear as bands of mineralization occurring distal to and separated from the irregular and frayed metaphyses. There is then gradual filling in of the demineralized area on the diaphyseal side of the initial band of mineralization with remodeling and the development of a normal trabecular pattern. Periosteal new bone formation may be seen, which gradually becomes incorporated into the cortices of the long bones.

The radiographic features of child abuse can sometimes be confused with nutritional rickets. The classic metaphyseal fractures of child abuse are “bucket handle” linear densities or triangular “corner fractures” from the lateral aspect of the metaphysis, which generally do not occur in rickets [251]. Fractures were present

in 17.5% of 45 US children under age 24 months with rickets and exclusively found in mobile infants and toddlers [252].

Measurement of bone density is not generally indicated for the evaluation of nutritional rickets. Nutritional rickets results in increased forearm bone area and reduced areal bone mineral density (BMD), which are more pronounced in the diaphyseal than in the metaphyseal regions of the radius and ulna, consistent with secondary hyperparathyroidism, generalized osteoid expansion, and impaired mineralization [253]. Nutritional rickets in Nigerian children was not associated with maternal forearm areal BMD [254]. After treatment of rickets, diaphyseal areal BMD z-scores did not differ between children with treated rickets and control children without rickets [255]. However, metaphyseal areal BMD z-scores were greater in children with treated rickets than in control children without rickets.

## 8. The growth plate in rickets

The characteristic feature in all forms of rickets is the abnormality that occur at the growth plate or physis. The cartilaginous plate consists of resting, proliferating, and hypertrophic zones of chondrocytes, and in rickets, it is the hypertrophic zone that is affected through widening and loss of organization of this zone. The widening is caused by marked impairment of apoptosis of the hypertrophied cells through an inhibition of the caspase-9-mediated mitochondrial pathway [256]. It appears that in all forms of rickets, the inhibition of apoptosis is as a consequence of hypophosphatemia, rather than through a direct effect of vitamin D or one of its metabolites [257,258]. Studies suggest that the sodium-dependent phosphate cotransporter (NaPi-IIc) is intimately involved as a phosphate transporter in hypertrophied chondrocytes [258], and thus as a regulator of apoptosis. The widening of the hypertrophic zone of the growth plate due to impaired apoptosis can be reversed or prevented by correcting the hypophosphatemia without altering the basic defect causing the rickets [256] (e.g., without altering PHEX or FGF23 in X-linked hypophosphatemia, or without correcting the VDR abnormalities in hereditary vitamin D-resistant rickets). However, correction of the hypophosphatemia does not correct the subgrowth plate metaphyseal changes of rickets, which manifest with impaired vascular invasion and reduced osteoclast numbers.

## 9. Treatment

Vitamin D-deficient rickets can be effectively treated by the oral administration of small doses of either

**TABLE 63.1** Recommended vitamin D<sub>3</sub> doses for the treatment of vitamin D deficiency in children of different ages.

Age	Daily dose for 90 days, IU	Single dose, IU	Maintenance daily dose, IU
<3 months	2000	N/A	400
3–12 months	2000	50,000	400
>12 months to 12 year	3000–6000	150,000	600
>12 year	6000	300,000	600

N/A, not available. Reassess response to treatment after 3 months as further treatment may be required. Ensure a daily calcium intake of at least 500 mg. For conversion from IU to µg, divide by 40.

Reproduced with permission from Ref. [22].

vitamin D<sub>2</sub> or D<sub>3</sub>, provided there is no evidence of gastrointestinal malabsorption. Stanbury et al. [195] showed that an oral vitamin D dose of between 200 and 450 IU/day produced a rise in serum 1,25(OH)<sub>2</sub>D concentrations to normal values within 1–3 days. The latter climbed to reach a peak some five times the normal mean after 1–3 weeks, despite serum 25(OH)D values remaining less than 10 ng/mL (25 nmol/L). Similarly, spontaneous improvement in the biochemical features of rickets has been reported to occur in children with biochemical abnormalities during the summer months, associated with a rise in serum 25(OH)D values due presumably to increased ultraviolet light exposure [259].

As per the Global Consensus guidelines on nutritional rickets, the recommended dose of cholecalciferol is 2000 IU/day in patients less than 1 year of age, 3000–6000 IU/day in patients aged 1–12 years, and 6000 IU/day in patients older than 12 years for 3 months (Table 63.1) [22]. A single large oral dose has also been used successfully, especially when caregiver adherence to the daily dose may be problematic.

Normalization of serum calcium and phosphorus concentrations occurs within 1 and 3 weeks [206], although serum alkaline phosphatase concentrations and urinary hydroxyproline excretion remain elevated for several months. Despite the return to normal of serum PTH, calcium, and phosphorus values within 3 weeks, serum 1,25(OH)<sub>2</sub>D concentrations may remain elevated for up to 10 weeks [201,206]. Serum 24,24(OH)<sub>2</sub>D values, which are often undetectable in the untreated patient, rise with the progressive increase in serum 25(OH)D concentrations during treatment [201]. Lower doses of vitamin D (1000–2000 IU/day) do produce healing, but the response is less rapid.

A randomized clinical trial found that daily vitamin D<sub>2</sub> or D<sub>3</sub> (2000 IU) was as effective as weekly vitamin D<sub>2</sub> (50,000 IU) over a 6-week period in correcting hypovitaminosis D in young children [260]. The authors concluded that the option of daily or weekly treatment may help to individualize treatment to suit parental needs and thus reduce the likelihood of noncompliance,

which has been reported to be a common problem especially when vitamin D supplementation is used long-term for the prevention of hypovitaminosis D. The study also clearly showed the equivalence of vitamin D<sub>2</sub> and D<sub>3</sub> in the treatment of hypovitaminosis D, a finding confirmed by Holick and coworkers, who measured serum 25(OH)D concentrations and found similar values in the D<sub>2</sub>- and D<sub>3</sub>-treated groups in adult subjects treated with either vitamin D<sub>2</sub> or D<sub>3</sub> at daily doses of 500 IU or 1000 IU over an 11-week period [261]. This equivalence may not hold when vitamin D is given in intermittent large doses as vitamin D<sub>2</sub> has a reduced half-life compared with vitamin D<sub>3</sub>.

In Central Europe, a single dose of 600,000 IU vitamin D (either orally or intramuscularly) has been found to be effective in treating vitamin D-deficient rickets, resulting in a rapid improvement in biochemical abnormalities within a few days and radiologic evidence of healing within 2 weeks [15,262]. Similarly, single-dose treatment with 600,000 IU has been used to treat rickets in the United States and India [262,263]. A sustained drop in serum alkaline phosphatase is seen within 6–12 weeks [262]. Single-dose therapy has an advantage over smaller daily doses as it avoids the problem of compliance, which was thought to be responsible for the lack of response in 40% of children with vitamin D-deficient rickets in a study conducted in Kuwait [264]. Concern has been raised about the use of 600,000 IU vitamin D in the treatment of rickets as hypercalcemia has been reported in a small number of infants a month after having received the vitamin D dose [265,266]. These authors suggest that a dose of 150,000 IU is equally effective as the larger dose in the management of the disease without running the risk of hypercalcemia. In a study conducted in Pakistan, the authors compared the efficacy of using either intramuscular or oral vitamin D<sub>3</sub> (200,000 IU as a single dose) for the treatment of active rickets in young children. No difference in response rate, which was very good, was noted between the two groups; however, the parents preferred the intramuscular treatment [267]. A single intramuscular dose of



vitamin D<sub>3</sub> (10,000 IU/kg body weight) has also been reported to be effective and safe in treating rickets in children [268]. 25(OH)D concentrations were above 20 ng/mL (50 nmol/L) in all but 12.5% of the subjects 3 months after the injection.

Besides ensuring an adequate vitamin D intake, the calcium content of the diet should be optimized (between 500 and 1000 mg/day). This is particularly true for children who are on vegetarian or low calcium-containing diets [269] and for those who are severely hypocalcemic. In symptomatic patients, a single dose of calcium gluconate (1–2 mL/kg of a 10% solution) may be given slowly intravenously and the diet supplemented with 10% calcium gluconate (5 mL/kg/day in divided doses). Oral calcium of 500 mg/day regardless of age or weight is recommended to prevent “hungry-bone syndrome” [22]. The combination of vitamin D and calcium in the treatment of nutritional rickets led to more rapid improvement in radiographic severity and alkaline phosphatase than either vitamin D or calcium alone [270,271].

Surgical correction of persistent leg deformities may be required in older children or in those with severe bone deformities. Most deformities of rickets correct spontaneously after medical treatment, without bracing or surgical intervention. In Indian children, the average rate of spontaneous correction was 1.9°/month with varus deformity and 0.92°/month with valgus deformity [272]. These investigators observed that varus deformities in children over the age of 4 years and 18° or greater valgus deformity in children over the age of 9 years usually do not correct spontaneously and may require surgical intervention. In Egyptian children with rickets treated with femoral and/or tibial temporary hemiepiphysiodesis at a mean age of 3.8 years, the mean time for resolution of deformity was 1.5 years [273]. Surgery was recommended for persistent or progressive tibiofemoral angle greater than 20° over 6 months.

## 10. Prevention

Vitamin D-deficient rickets remains a problem in at-risk groups despite readily available methods of preventing the disease. Prospective studies have assessed vitamin D status in breastfed infants in multiple countries. Several have shown a fall in serum 25(OH)D concentrations in those infants who were not vitamin D supplemented, to levels in the vitamin D deficient range [30,274,275], although this was not a universal finding [276,277]. Further, a number of studies have highlighted the high prevalence of vitamin D deficiency in mothers during pregnancy and lactation, which exacerbates the severity and onset of vitamin D deficiency in their offspring [278–281]. In a number of countries, a further

group that probably does not receive enough attention is the adolescent, as the prevalence of vitamin D deficiency tends to rise in this age group [282,283]. In the United States, adolescent vitamin D deficiency is particularly prevalent in African American teenagers, in the overweight, and in females [284].

Thus, preventive strategies should be directed not only at breastfed infants but also at pregnant and breastfeeding women and adolescents where appropriate [285]. North America [19] recommends dietary intakes of vitamin D between 600 and 1200 IU/day for pregnant and lactating women, respectively, to ensure adequate circulating 25(OH)D levels. In 2016, the Scientific Advisory Committee on Nutrition in the United Kingdom [286] did not recommend an increased vitamin D intake in pregnant and lactating women over and above the standard 400 IU/day to maintain 25(OH)D > 25 nmol/L. Yet, studies have shown that these recommended intakes may not be adequate to maintain 25(OH)D concentrations in the sufficiency range [68,287–289], and some researchers suggest that intakes of 1000–2000 IU/day are more appropriate [290].

Although at normal circulating maternal 25(OH)D concentrations, the vitamin D content of breast milk is limited, maternal supplementation with vitamin D at 4000–6400 IU/day increases breast milk vitamin D concentrations sufficiently to maintain the infant's 25(OH)D within the normal range [67,291]. Most breastfeeding mothers in a US practice preferred supplementing themselves rather than their infants, and most preferred daily rather than monthly supplementation [292]. Safety was most important to mothers in choosing a method of supplementation. Taking maternal preferences into consideration for infant or maternal vitamin D supplementation may help to ensure adequate intakes of vitamin D in breastfed infants. Potential advantages of maternal over infant supplementation include ease of administration, simultaneous mother and infant supplementation, and lower risk of toxicity to the infant from dosing errors. Oral vitamin D 120,000 IU monthly given to lactating mothers in India achieved 25(OH)D > 20 ng/mL in 95% of infants [293]. Monthly maternal supplementation could be incorporated in established mother/child visits for prevention of nutritional rickets.

North America and the United Kingdom recommend vitamin D dietary intakes of 400 and 340–400 IU, respectively, for the breastfed infant [19,286]. The Global Consensus Guidelines for the Prevention of Rickets recommended that all infants, irrespective of feeding methods, receive daily vitamin D of at least 400 IU for the first 12 months of life, and 600 IU should be given to pregnant women, toddlers, and children at high risk of vitamin D deficiency and rickets [22]. The American Academy of Pediatrics advises that infants less than 6 months of age should be kept out of direct sunlight,



that children's activities should minimize sunlight exposure, and that sunscreens should be used, because of the indirect evidence that early exposure to sunlight might determine the risk of skin cancer in later life [294]. These recommendations make it imperative that if the aforementioned guidelines are followed, supplemental vitamin D (400 IU/day) should be provided to all breastfed and weaned infants ingesting less than 500 mL of fortified infant milk formula/day [295–298]. In a prospective study conducted in China on infants from birth to 6 months of age, supplemental vitamin D at a dose of 400 IU/day produced more normal circulating 25(OH)D concentrations than did either 100 or 200 IU/day [299]. However, in a small number of infants, even 400 IU/day did not maintain 25(OH)D levels above 11 ng/mL (27.5 nmol/L). Nevertheless, no radiologic evidence of rickets was found in any of the infants in the three groups at 6 months of age [87,300]. Although 400 IU/day of vitamin D prevents rickets and maintains a normal vitamin D status in the majority of infants [301], higher intakes may be required to reduce the prevalence of type 1 diabetes in children [302].

Infants who are fed milk formulas or cow's milk fortified with vitamin D and drink more than 1000 mL/day do not require vitamin D supplements, as their intake of milk generally provides sufficient vitamin D to prevent deficiency [294]. The recent consensus statement [22] does not differentiate between breastfed and artificially fed infants, as no harmful effects of the additional vitamin D intake in artificially fed infants have been reported.

High single dose therapy (stosstherapie) has been used with success in the treatment of vitamin D-deficient rickets in a number of countries. A similar dosing strategy has also been used on a regular intermittent basis of every 3–5 months for the first 18 months of life for prevention of vitamin D deficiency. In a study assessing the effect of high doses of vitamin D (600,000 IU) on calcium and vitamin D metabolism in infants [15], serum 25(OH)D concentrations reached very high levels 2 weeks after each administration, but these had returned to normal prior to the next dose. 1,25(OH)<sub>2</sub>D generally remained within the normal range; however, 34% of infants were hypercalcemic at some stage during the study. These results led the authors to conclude that the dosage regimen as used during the study was excessive and unsafe [15]. However, a study, using the same dose (600,000 IU intramuscularly) annually in adults for the management of vitamin D deficiency, reported that at the end of 12 months, serum 25(OH)D concentrations were still in the optimal range and that the treatment appeared safe, although further studies are required to examine urine calcium excretion and the risk of hypercalcemia in a larger group of subjects [303].

Following on these results, a study to assess the efficacy of a single dose of vitamin D (600,000 IU or 15 mg) at 15 days of life, compared with 200,000 IU (5 mg) or 100,000 IU (2.5 mg) at birth and three monthly for 9 months, has been undertaken [304]. Two weeks after the initial administration, 28 of 30 infants in the 15 mg group had serum 25(OH)D concentrations above the upper limit of normal (mean  $\pm$  SD for the group;  $307 \pm 160$  nmol/L) compared with 58% ( $150 \pm 55$  nmol/L) in the 5 mg group and 23% ( $92 \pm 42$  nmol/L) in the 2.5 mg group. At 6 months of age, 50% of the infants who had received 15 mg at birth still had elevated 25(OH)D concentrations (defined in this study as being  $>120$  nmol/L), while in the 5 mg group, none had elevated levels. In the group receiving 2.5 mg three monthly, serum 25(OH)D values were in the normal range on each occasion prior to receiving the next dose. Although hypercalcemia was not detected in any of the infants, serum calcium concentrations were higher in the 15 mg group 2 weeks after receiving the dose than in the other two groups. The authors concluded that intermittent doses of 15 mg vitamin D during the first year of life are excessive and that 5 mg every 6 months or even better 2.5 mg every 3 months are more suitable for the prevention of vitamin D deficiency in at-risk infants.

The same group of researchers studied the use of intermittent vitamin D supplementation to prevent vitamin D deficiency during the winter months in adolescents in northern France [305]. They recommend a single dose of 100,000 IU in early autumn, midwinter, and again at the beginning of spring to maintain 25(OH)D concentrations above 20 ng/mL. On this regimen, the usual winter rise in PTH and fall in vitamin D status were prevented. However, in a well-designed randomized controlled trial of vitamin D<sub>3</sub> 100,000 IU orally every 3 months for 18 months in infants in Afghanistan, the prevalence of rickets was similar in the vitamin D and control groups at the end of the study (5.3% vs. 5.5%, respectively) [306]. Height-for-age z-scores were also similar between the two groups at the end of the study. Concern with high-dose intermittent therapy has been raised, related to the observation that a single high dose of vitamin D can induce 24-hydroxylase (CYP24A1), resulting in greater catabolism of 25(OH)D through increased production of 24,25(OH)<sub>2</sub>D relative to 1,25(OH)<sub>2</sub>D compared with that seen during daily supplementation [181,307,308].

Vitamin D supplementation should be considered for all infants until they are ambulatory and are able to play outside [309]. Even in countries close to the equator, where sunlight exposure should not be a problem, social customs may place the mother and infant at risk from vitamin D deficiency. In such situations (e.g., the Middle East and in Muslim communities in North Africa and

the Asian subcontinent), vitamin D supplementation may also be necessary to reduce the high prevalence of vitamin D deficiency [279,310].

In some countries, vitamin D deficiency is not just a finding in breastfed infants and their mothers. Rickets has been described in adolescents of Indian and Pakistani descent in the United Kingdom and in the Middle East [311,312], while hypovitaminosis D has been reported in adolescents in a number of European countries [313–316], India [317,318], and China [319]. To address this problem, Fuleihan and her coworkers have studied the use of weekly therapy over 1 year in adolescent schoolchildren [320]. Vitamin D<sub>3</sub> 14,000 IU weekly produced mean serum 25(OH)D concentrations in the mid-30s (ng/mL), while a dose 10 times smaller (1400 IU weekly) did not raise the mean above 20 ng/mL (50 nmol/L) in the female subjects. They concluded that a vitamin D dose of 14,000 IU/week (equivalent of 2000 IU/day) was safe and effective in ensuring vitamin D sufficiency in an adolescent population at risk of vitamin D deficiency.

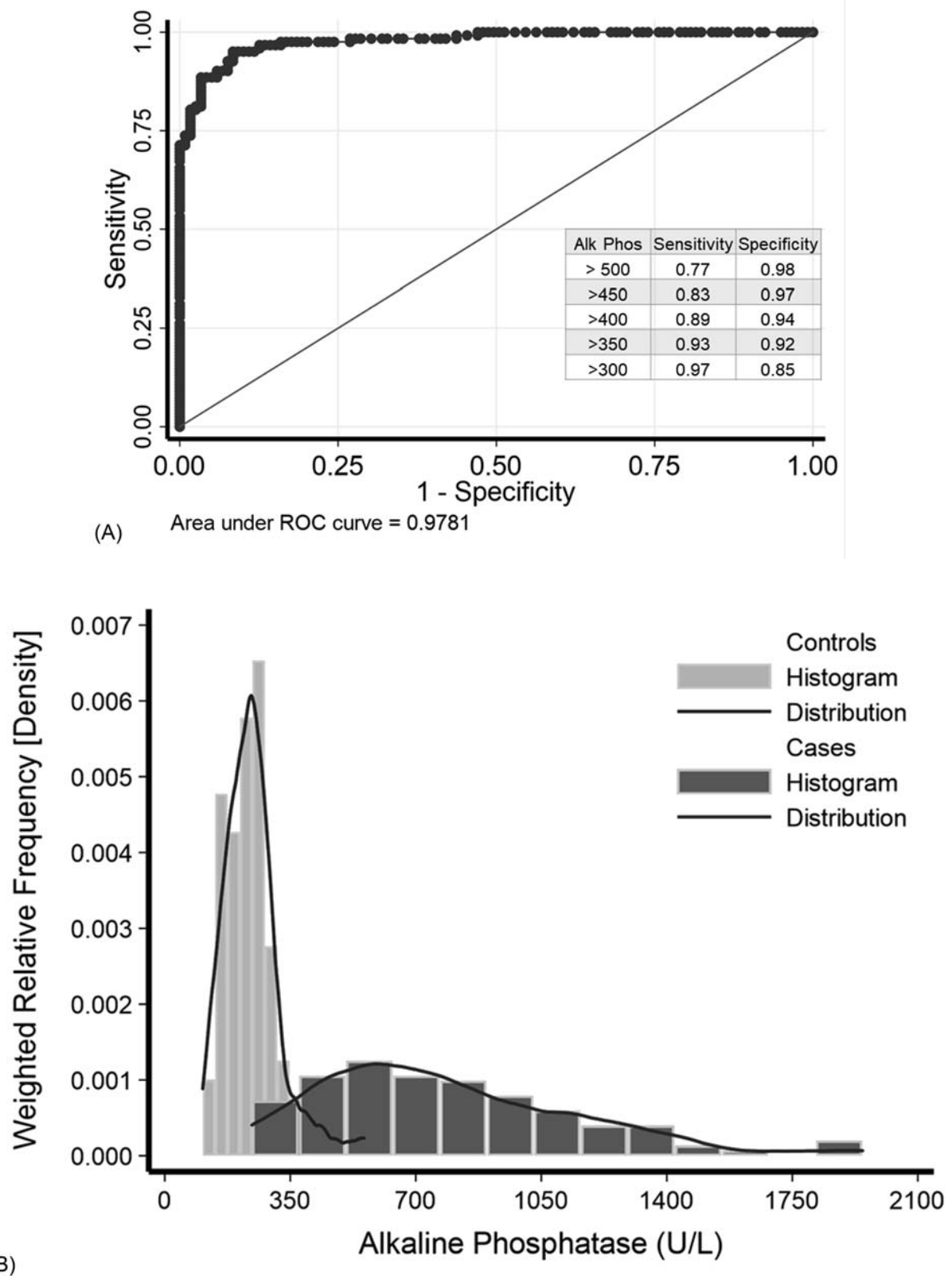
With widespread vitamin D deficiency in many countries, vitamin D supplementation is unlikely to be an effective means of combating the disease on a community basis, and food fortification should be considered a complementary solution [321].

Nutritional rickets and its consequences are preventable global public health problems [322]. The clinical manifestations are the “the tip of the iceberg,” indicating widespread vitamin D and/or calcium deficiencies [138]. Prevention programs can be targeted to reach high-risk groups of the population, or they can use an universal approach to cover all members of the population. Combining both approaches is most effective. Public health interventions first require assessment of the disease burden and the magnitude of the problem, as prevention programs have their greatest impact in populations with a high prevalence of nutritional rickets. The burden of nutritional rickets is measured both by its prevalence and by its health consequences. The prevalence of nutritional rickets is greatest in Asia, the Middle East, and Africa. In high-income countries, the disease burden is greatest in ethnic minority, immigrant, and refugee populations. Case ascertainment of nutritional rickets requires a uniform case definition, based on clinical features, followed by confirmatory radiographs, which are the gold standard for diagnosis of active rickets. Consensus is needed to determine the best methods to estimate the prevalence of rickets, particularly in communities with limited resources [323]. Clinical signs of rickets have been used extensively, but are likely to overestimate the prevalence and depend on clinical skills. Population screening for rickets utilizing the measurement of serum 25(OH)D or radiographs is not recommended [22,324]. Alkaline

phosphatase measurements have also been considered as a biomarker of active rickets, but the specificity of elevated levels indicative of rickets depends on the underlying prevalence of active rickets in the community. Recently a study from Nigeria has highlighted the good discriminatory value of an elevated alkaline phosphatase value to differentiate those children with active rickets from community controls [216] (Fig. 63.4). Because the incidence of nutritional rickets is an indicator of vitamin D and calcium deficiency within the population, rickets surveillance in low- and middle-income countries would highlight areas in greatest need of a vitamin D status survey or intervention [325].

Nutrient supplementation is the fastest way to improve the micronutrient status of individuals or targeted populations [324]. Programs that universally supplement infants with vitamin D 400 IU/day from birth to 12 months of age have prevented nutritional rickets in healthy infants who have an adequate calcium intake [126,299,300]. Successful vitamin D supplementation in Turkey resulted from training parents how to give vitamin D, explaining the benefits, and continuous monitoring and evaluation [87,300]. As a result of this program, the prevalence of rickets declined from 6% to 0.1%, demonstrating the success of an infant vitamin D supplementation program. Beyond 12 months, children at risk should continue vitamin D supplements. Vitamin D supplementation is best integrated into existing childhood primary health and antenatal care programs, which already provide recommended micronutrients and immunizations. Daily vitamin D supplementation of infants effectively improves their vitamin D status [326,327], but adherence is the primary obstacle with this approach. Single large doses of oral vitamin D, given to infants as part of an immunization program, provide a good opportunity to ensure that children receive adequate vitamin D (for example, 50,000 IU every 2 months [325,328]). This approach is similar to the administration of high-dose vitamin A supplementation in low-income countries to reduce morbidity from measles. The effects of a single dose of vitamin D can last for 3 months or more, and this approach may be preferred when adherence to daily supplementation is problematic.

Food fortification is a universal strategy that reaches an entire population. Food fortification has a less immediate, but ultimately a wider and more sustained impact than supplementation programs, and fortification is generally more cost-effective than other interventions [325]. Vitamin D is naturally found in only a limited number of foods, and apart from food fortification, dietary intakes have little impact on vitamin D status. Fortifying commonly consumed staple foods with vitamin D and calcium based on dietary patterns safely provides adequate intake to prevent deficiency at minimal cost.



**FIGURE 63.4** The utility of serum alkaline phosphatase in diagnosing active rickets in children with suspected dietary calcium deficiency using a case-control study. The controls were age- and sex-matched with the cases of active rickets, which were diagnosed using radiographs of the knees and wrists. (A) is a plot of the receiver operating characteristic curve plotting true positive rate (sensitivity) against false positive rate (1-specificity). (B) depicts the alkaline phosphatase values in cases and controls. *Reproduced with permission from Ref. [216].*

Food fortification effectively prevents nutritional rickets and improves the vitamin D status of children [329,330]. Following vitamin D fortification of milk in North America and of milk, margarine, and cereals in the United Kingdom, the prevalence of rickets dramatically declined, so much so that it was considered almost eradicated [331].

Although the untargeted fortification of foods other than milk and infant milk formulas has been used in the past, the problems of hypercalcemia experienced in the United Kingdom after World War II led to it falling into disfavor (as discussed earlier in this chapter). However, many of these cases of infantile hypercalcemia were likely to be due to impaired metabolism of vitamin D (inactivating mutations of the *CYP24A1* gene encoding the 24-hydroxylase enzyme) rather than to excessive vitamin D alone [332]. Women with an inactivating *CYP24A1* mutation are also at risk for hypercalcemia in pregnancy [333,334]. Targeted food fortification has been studied in the Asian community in Great Britain as a means of reducing the high prevalence of vitamin D deficiency in both adults and children in that community [335]. Fortification of chapatti flour at a level of 6000 IU/kg for 6 months produced a sustained and significant rise in serum 25(OH)D concentrations comparable with those achieved by a weekly dose of 3000 IU vitamin D. Serum calcium and phosphorus values rose, and the number of subjects with biochemical abnormalities suggestive of rickets fell. The authors concluded that fortification of chapatti flour is a cheap and effective method of preventing vitamin D deficiency in the Asian community in Britain and has the advantage over daily or intermittent vitamin D supplementation as adherence to supplementation is often poor. Fortification of widely consumed food vehicles is most effective when the program is mandatory and enforced [325]. Nevertheless, food fortification can be a controversial public issue. In the United States not only have there been isolated reports of vitamin D toxicity related to inadequate monitoring of the fortification process [14], but underfortification can also be a problem [336].

Low dairy product intake, a common situation in low-income countries, is a risk factor for nutritional rickets from inadequate dietary calcium in children over the age of 12 months. In areas with inadequate calcium intakes, indigenous food sources of calcium or food fortification with low-cost calcium carbonate can be promoted or subsidized [104,337]. Successful food fortification requires selection of culturally appropriate and affordable staple foods [338,339]. Government regulation is necessary to establish food fortification requirements and monitor the fortification process.

Because correction of vitamin D deficiency is relatively easy, inexpensive, and cost efficient through fortification, there have been multiple calls to eradicate

rickets globally through fortification programs [22,325,340]. Fortification of habitually consumed foods is the most effective means of assuring adequate vitamin D status and prevention of rickets at a population level. Food fortification and supplementation programs are synergistic interventions [341]. A review of food-based solutions for preventing vitamin D deficiency, such as fortification and supplementation, found that 25(OH)D values > 50 ng/mL (125 nmol/L) were rare, and no adverse events were observed [342]. There are limited data regarding vitamin D intake and exposure in LMICs, and improving these estimates will aid in identifying countries where a 25(OH)D assessment survey is necessary [325].

The effectiveness of public health interventions can be assessed by determining adherence to recommended vitamin D and calcium intakes and by case surveillance for nutritional rickets. Periodic nutrition surveys of dietary intakes and vitamin D status enable policy-makers to assess the level of population risk for nutritional rickets. A Cochrane Review concluded that the effects of preventive measures for nutritional rickets should be investigated in different countries, different age groups, and in children of different ethnic origins [343]. Assessing the micronutrient status of populations; monitoring and evaluating the impact of strategies for the prevention and control of micronutrient malnutrition; and tracking related trends over time are included in the mandate of the WHO [344].

## 11. Dietary calcium deficiency

Conventional wisdom would have it that nutritional rickets is primarily due to vitamin D deficiency, although dietary calcium intake modulates the severity and rapidity of onset of the disease [345,346]. However, accumulating evidence implicates low dietary calcium intakes as a cause of rickets in the face of serum 25(OH)D concentrations above values generally associated with vitamin D-deficient rickets (<25 nmol/L).

Isolated case reports of rickets developing in infants and toddlers, who were placed on very low calcium diets, have been published [347–350]. Their clinical and biochemical presentations were very similar to those of infants with vitamin D deficiency; however, in three of the five infants, serum 25(OH)D and 1,25(OH)<sub>2</sub>D values were reported to be greater than 9 ng/mL (22.5 nmol/L) and 118 pg/mL (295 pmol/L), respectively. In none of the five infants was a therapeutic trial of calcium supplementation of the diet alone tried; however, the clinical and biochemical presentation suggested to the authors that dietary calcium deficiency was the primary factor responsible for the development of rickets.



More convincing evidence of dietary calcium deficiency as a cause for rickets in children comes from studies in South Africa [351,352], Nigeria [353–356], India [105,317], and Bangladesh [106,357], where the staple diets of children are characteristically low in calcium because of the lack of readily available dairy products and the low calcium content of the cereals (maize [corn], cassava, yam, rice, and plantain) [92,138,358,359]. In the South African children, dietary calcium intakes have been estimated to be between 90 and 300 mg/day in those children suffering from rickets compared with between 200 and 500 mg/day in age-matched controls [358], while in the Nigerian children, both patients and controls had similar but very low calcium intakes (200 mg/day) [167]. In children with active rickets in Bangladesh, mean calcium intakes were 156 mg/day compared with 323 mg/day in controls [357].

In South Africa, the children typically came from rural areas and presented with signs and symptoms of rickets between the ages of 4 and 15 years (Fig. 63.4) [360], while in Nigeria, they presented younger, at a mean age of approximately 4 years [354]. In the South African series, half the children presented with knock-knees, while the others presented with either bow-legs or wind-swept deformities (Fig. 63.5). Bow-legs were more common in the Nigerian children, probably reflecting their earlier age of presentation [167]. Unlike vitamin D deficiency, symptoms of muscle weakness were characteristically absent in older children with dietary calcium deficiency.

Radiologically, the features are typical of calciopenic rickets with osteopenia and features of hyperparathyroidism being frequent findings. The severity of the metaphyseal changes is variable (Fig. 63.6). Older children (teenagers) may have no radiologic changes of rickets despite features of osteomalacia on the iliac crest bone biopsy [361]. Younger children may show evidence of only minor degrees of impaired endochondral calcification, while in others the metaphyseal changes may be quite marked. In a Nigerian study, the degree of severity of radiologic rickets was correlated with serum alkaline phosphatase values [214].

The biochemical features are similar to those of other causes of calciopenic rickets. Hypocalcemia, low urinary calcium excretion, and elevated serum PTH and alkaline phosphatase concentrations are characteristics, while serum phosphorus values are variable and may be within the reference range for age [92,138,357,359]. Serum 25(OH)D values tend to be lower in those with active rickets than controls, but are often above values generally considered to be associated with vitamin D deficiency (mean 16.4 ng/mL (41 nmol/L), 14.4 ng/mL (36.0 nmol/L), and 9.5 ng/mL (23.7 nmol/L) in the South African, Nigerian, and Bangladeshi children,

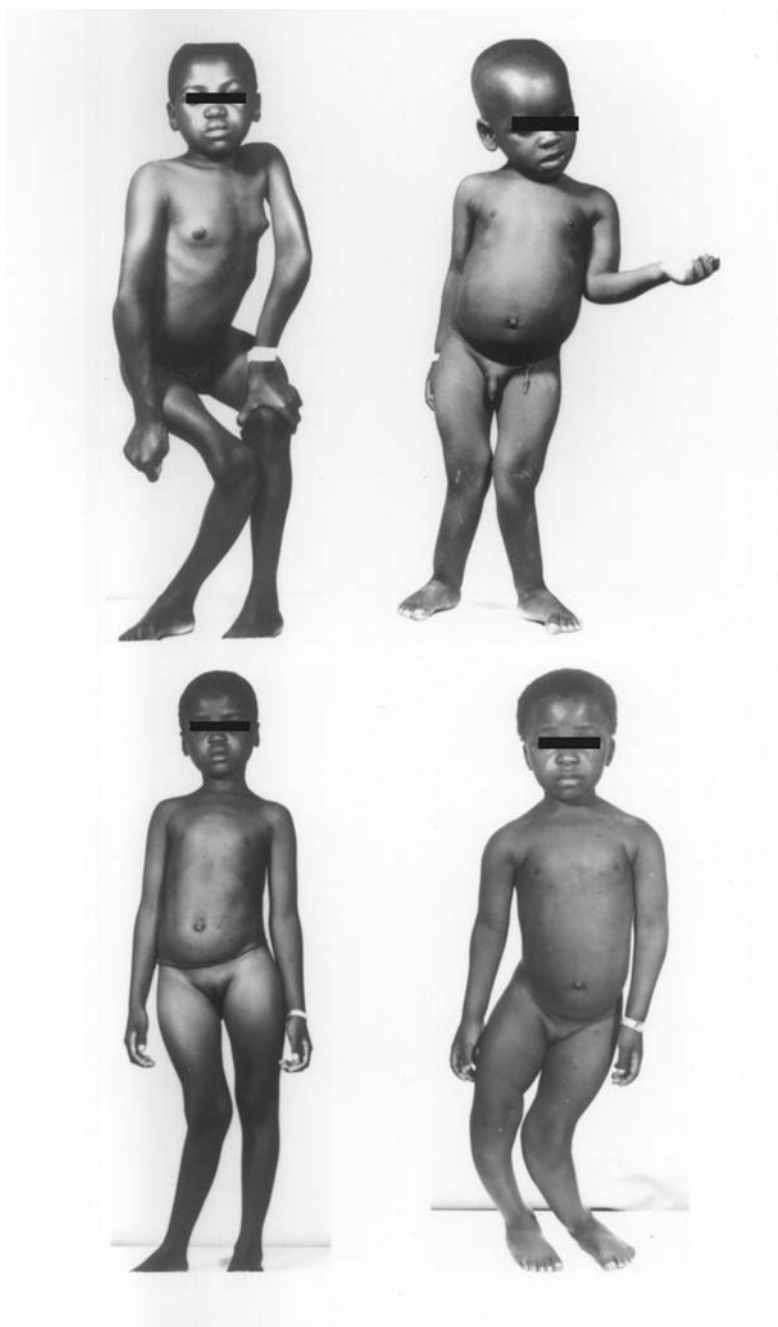
respectively), and 1,25(OH)<sub>2</sub>D concentrations are markedly elevated [354,355,362]. In a Nigerian study [92] and in the South African children [221], serum osteocalcin levels were similar to those of nonrachitic controls in the majority of patients, although another report from Nigeria found slightly higher levels in rachitic patients than controls [210].

In a small number of children who had iliac crest bone biopsies, they revealed evidence of osteomalacia and hyperparathyroidism in those children who have radiologic features of rickets [352], while in the teenagers without radiologic changes but lower limb deformities, the histologic picture varied from that of decreased bone volume, through features of hyperparathyroidism, to frank osteomalacia associated with hyperparathyroidism [361].

In both the Nigerian and South African studies, clinical, biochemical, and radiologic healing has been achieved through increasing the calcium intake of the children to between 800 and 1500 mg/day without the administration of vitamin D supplements [92,351]. A study using a calcium supplement of only 350 mg/day reported complete healing within 6 months [356]. A study in Nigeria showed that a daily elemental calcium dose of 1000 mg was associated with more rapid healing than 500 mg/day, but the use of 2000 mg/day did not have greater benefit than 1000 mg [363]. In a randomized controlled trial, calcium supplements alone or with vitamin D were equally effective in healing the bone disease and were significantly better than vitamin D therapy alone [354]. However, other studies suggest that combining vitamin D with calcium may result in improved healing of rickets, even when caused by dietary calcium deficiency [271,364]. In the majority of the South African children, orthopedic corrective surgery has been necessary to correct the deformities of the legs once biochemical and radiologic healing has occurred. This has not been the pattern in the younger Nigerian children with rickets, who have shown remarkable modeling and straightening of deformities without orthopedic surgical intervention [356] (Fig. 63.7). Similarly, studies from Bangladesh suggest that early medical treatment reduces the number of children requiring corrective surgery [106].

The data available from epidemiologic studies conducted in a rural area in South Africa in which a number of the affected children live, suggested that asymptomatic dietary calcium deficiency was prevalent in schoolchildren living in the area. Some 13% of children between the ages of 7 and 12 years were hypocalcemic, 41.5% had elevated alkaline phosphatase concentrations, and 76% had low urinary calcium excretion [365]. It is unclear if these children have long-term sequelae because of the poor calcium intakes. However, studies do indicate that asymptomatic children with





**FIGURE 63.5** The clinical presentation of children with dietary calcium deficiency. The deformities are typically more severe in the legs with a predominance of knock-knees or windswept deformities. Upper limb deformities are usually mild if present at all. *Reproduced with permission from Ref. [360].*

biochemical abnormalities living in the rural community have lower appendicular bone mass than those with normal biochemistry [358] and that children in the community as a whole have lower appendicular bone mass than their urban peers [366].

Although dietary calcium intakes in children with biochemical changes suggestive of dietary calcium deficiency are very low, it is unclear what role the high

phytate or oxalate contents of the diet play in aggravating the symptoms. Nevertheless, biochemical improvement can be achieved by supplementing the children with 500 mg calcium daily [367]. The finding of similarly low dietary calcium intakes in patients with rickets and age-matched controls in Nigeria is intriguing [167], as it suggests that factors other than low dietary calcium intakes might influence the



**FIGURE 63.6** The radiographic features of dietary calcium deficiency rickets in the lower limbs of a child. The long bones are osteopenic with deformities characteristic of long-standing rickets. The metaphyses show evidence of impaired mineralization and growth arrest lines, and the physes of the femurs and tibias are widened and irregular.

development of rickets in affected children. Such factors might include differing amounts of inhibitors of calcium absorption in the diet, differing vitamin D status [137], differing growth rates and therefore calcium requirements in the children, or genetic differences that make the rachitic children less able to adapt to low dietary calcium intakes than control subjects. A number of these factors are currently under investigation. A small study has found that there are significant differences in the frequency of vitamin D receptor polymorphisms between affected and control children with the *FF* genotype being

more common in children with rickets, but the significance of these findings is unclear at present [47]. One of the hallmarks of dietary calcium deficiency, which differentiates it from vitamin D deficiency, is the markedly elevated serum concentrations of  $1,25(\text{OH})_2\text{D}$  in untreated subjects. These values are even higher than those found in age-matched controls, who had dietary calcium intakes similar to those who developed rickets [167]. In a situation of low dietary calcium intake, it would be expected that these high  $1,25(\text{OH})_2\text{D}$  concentrations would maximize intestinal calcium absorption. Studies using

stable isotopes of calcium have confirmed this hypothesis in children with active rickets in Nigeria [231,368]. The mean intestinal fractional calcium absorption of just over 60% in these children is much greater than that reported in subjects with vitamin D deficiency (10%–15%) [369] and is in keeping with the primary pathogenetic mechanism being dietary calcium lack rather than vitamin D deficiency.

1,25(OH)<sub>2</sub>D is the primary factor regulating intestinal calcium absorption and thus is responsible for the acquisition and preservation of mineralized bone mass [370]. 1,25(OH)<sub>2</sub>D shifts calcium from bone to serum during a negative calcium balance. Hogler has emphasized that low vitamin D or low calcium intake alone does not cause problems; however, rickets can develop if both are insufficient or deficient as shown in Fig. 63.1 [371]. A recent reanalysis of the data obtained from studies in Nigeria has highlighted the interaction between 25(OH)D levels and dietary calcium intake in the pathogenesis of rickets in children on low dietary calcium intakes [137] (Fig. 63.8). Calcium-deficient rickets causes secondary hyperparathyroidism and increased 1,25(OH)<sub>2</sub>D production which in The Gambia was associated with an increase in serum FGF23 [370]. 1,25(OH)<sub>2</sub>D production also stimulates the production of inhibitors of mineralization such as pyrophosphate

and osteopontin. Therefore, not only vitamin D deficiency but also an increase in 1,25(OH)<sub>2</sub>D from calcium deficiency impairs mineral deposition resulting in rickets [372]. It is possible that relative vitamin D insufficiency might play a role in children with presumed dietary calcium deficiency rickets, as 25(OH)D concentrations are lower in children with active rickets than in their age-matched controls [137,167]; however, the addition of vitamin D to the calcium supplements in a randomized controlled trial did not significantly improve the response of rachitic children to treatment [354]. Nevertheless, bolus administration of vitamin D (50,000 IU) to untreated rachitic children resulted in a rapid and marked rise in 1,25(OH)<sub>2</sub>D concentrations, which peaked on day 3 and then declined over the following 10 days [373]. These findings suggest that there might be relative substrate (25(OH)D) deficiency, which is corrected by the provision of the bolus of vitamin D. A more recent randomized controlled trial in Nigerian children with nutritional rickets from insufficient dietary calcium showed that children benefitted from both calcium, in the form of limestone, and vitamin D supplementation to promote more rapid healing of the rickets [271]. These and other study findings suggest that the prevention of nutritional rickets in high-risk populations should include fortification of staple foods



**FIGURE 63.7** The response to nonsurgical treatment of lower limb deformities in children with rickets due to dietary calcium deficiency. Treatment consisted of a combination of calcium and vitamin D supplements. (A) Child at the age of 2.7 years prior to commencement of treatment. (B) The same child following healing of the rickets. (C) Another child aged 4.5 years prior to treatment. (D) The same child following biochemical and radiological healing of the rickets.

with a combination of calcium (possibly in the form of limestone) and vitamin D since an increased intake of calcium protects individuals from the effects of vitamin D deficiency [137,374] and vitamin D supplementation may promote more rapid healing in calcium-deficient rickets [271].

## 12. Rickets of prematurity or metabolic bone disease of prematurity

Rickets or osteopenia of prematurity, also referred to as metabolic bone disease of prematurity (MBDP), is a reduction in bone mineral content presenting with a combination of biochemical abnormalities and/or radiological features of rickets in premature infants. Although not usually discussed in sections on nutritional rickets, there is no doubt that a major contributing factor for developing the disease is a relative deficiency of phosphate intake in very-low-birth-weight infants during the first 3 months of life. The incidence of MBDP is reported to be between 23% and 32% in very-low-birth-weight (<1500 g) and up to 50% in extremely low-birth-weight infants (<1000 g) [375,376]. The main risk factors for reduced bone mineralization are low gestational age and birth weight [375–378].

Approximately 80% of fetal bone mineral accretion occurs in the third trimester of pregnancy with 300 mg/day of calcium being transferred to the fetus between 35 and 38 weeks' gestation [379]. Calcium, phosphate, and magnesium are actively transported across the placenta from the maternal circulation even if maternal supplies are low. The high fetal–maternal gradient of minerals allows the fetus to have “hypercalcemia” (compared to maternal concentrations) to promote skeletal formation with low levels of parathyroid hormone (PTH), calcitriol, and sex steroids [380]. The calcium-sensing receptor maintains suppression of PTH for fetal hypercalcemia to be maintained [379]. High calcitonin levels found in the fetus also promote mineral deposition. Parathyroid hormone–related peptide (PTHrP) has an additive role in the regulation of mineral homeostasis and also stimulates bone formation [379]. Premature infants lack exposure to the third trimester intrauterine fetal environment during which period a greater growth and intake of minerals occur. The consequences of preterm birth increase the nutritional and metabolic stresses these neonates have to adapt to. Besides the reduced placental transfer of calcium and phosphate in preterm infants, these additional risk factors, often in combination, predispose these infants to develop MBDP. The ante- and postnatal risk factors for MBDP are listed in Table 63.2 [380].

**TABLE 63.2** Antenatal and postnatal risk factors of metabolic bone disease of prematurity.

Antenatal	Postnatal
Placental insufficiency	Prolonged total parenteral nutrition > 4 weeks
Preeclampsia	Bronchopulmonary dysplasia
Chorioamnionitis	Necrotizing enterocolitis
Males	Liver disease or severe cholestasis
Genetic polymorphisms (vitamin D receptor, estrogen, collagen alpha I)	Renal disease
Neuromuscular disorders	Medications (loop diuretics, methylxanthines, glucocorticoids)
Intraventricular hemorrhage Periventricular leukomalacia	Prolonged mechanical ventilation and immobilization

*Adapted with permission from Ref. [380].*

In addition to the contributory ante- and postnatal factors, the preterm infant is most likely to develop MBDP from suboptimal nutritional support in the postnatal period. Breast milk feeds are the ideal choice of nutrition but the gradual initiation of these feeds and the preterm infants' intestinal immaturity are rate limiting factors to the optimal absorption of calcium and phosphate. Due to the decreased intake and/or absorption of calcium and phosphate, the preterm infant needs special fortified formulas or breast milk in view of the low levels of phosphorus and calcium in breast milk [381,382]. The average absorption of dietary calcium is 60% in breastfed infants, and the amount can be increased by human milk fortifiers containing highly soluble calcium glycerophosphate [383,384]. Preterm infant's phosphorus intake is as low as 0.5 mmol/kg/day postnatally compared with 2.1–2.6 mmol/kg/day in utero [381]. It is important to consider that prolonged total parenteral nutrition (TPN) and early aggressive parental nutrition providing approximately 4 g/kg/day of intravenous protein in the first week of life has been associated with severe hypophosphatemia in neonates with birth weights <1250 g [385]. Calcium and phosphorus intake levels of 3.4 mmol/kg/day and 2.6 mmol/kg/day, respectively, with adequate protein intake are required to achieve a postnatal average weight gain of 17 g/kg/day (the fetal growth rate before 35 weeks of gestation). Any protein supplied above 2 g/kg/day will require an additional phosphorus intake to ensure adequately accretion. Protein intake of 3.5 g/kg/day promotes good extrauterine growth [385,386], but hypophosphatemia develops due to the infants' high metabolism and utilization of phosphorus [385].

Clinical features of MBDP such as splayed sutures, craniotabes, frontal bossing, widened wrists and



enlarged costochondral junctions or rachitic rosary with tachypnea, fractures, and postnatal growth failure can manifest between 3 and 12 weeks of age [387].

The routine baseline serum biochemical markers for screening should include calcium, phosphate ( $\text{PO}_4$ ), alkaline phosphatase (ALP), PTH, and a calculation of the renal tubular reabsorption of phosphate (TRP). The American Academy of Pediatrics Committee on Nutrition (AAP-CON) recommends screening all very-low-birth-weight infants for MBDP with serum  $\text{PO}_4$  and ALP starting at 4–5 weeks of age [388]. Thereafter, the assessment should include weekly or biweekly serum  $\text{PO}_4$  and ALP [389,390]. According to AAP-CON, an ALP level  $>500$  IU (depending on the assay methodology used) and  $\text{PO}_4$  levels  $<3.5$ – $4$  mg/dL ( $1.13$ – $1.29$  mmol/L) are suggestive of MBDP [388]. Kavurt et al. [391] studied VLBW infants who received total parental nutrition (TPN) from the first day of life; of the 254 infants, metabolic bone disease was diagnosed in 37%, 72 (28%) developing radiological evidence of rickets and 24 (0.9%) only biochemical abnormalities. In those with radiological evidence of rickets, the serum ALP was significantly higher and the serum  $\text{PO}_4$  level significantly lower than in those with no radiological changes. The serum calcium levels were similar and normal in both these groups [391]. However, no single value of ALP or  $\text{PO}_4$  was related to radiological findings of MBDP [392]. An X-ray of the wrist and/or knee is recommended in VLBW infants when two values of ALP measured at least 1 week apart exceed 800 IU/L [387]. Backström et al. reported that ALP levels  $>900$  IU/L and persistently low  $\text{PO}_4$  levels  $<5.6$  mg/dL ( $1.81$  mmol/L) in VLBW preterm infants  $<33$  weeks' gestation have a diagnostic sensitivity and specificity of 100% and 70%, respectively, to diagnose MBDP [393]. Hypophosphatemia is the primary biochemical alteration in MBDP causing a reduction in PTH and FGF23 secretion with an increase in TRP. The decrease in phosphate stimulates 1,25 dihydroxyvitamin D synthesis and increases intestinal calcium absorption causing hypercalcemia, hypercalciuria, and

nephrocalcinosis. Neonatal TRP is generally 78%–91%, and a value of  $>95\%$  is a significant marker of insufficient  $\text{PO}_4$  supplementation [389,394]. The TRP is calculated using the following formula:  $[1 - (\text{urinary phosphate/urinary creatinine} \times \text{serum creatinine/serum phosphorus})] \times 100$ . Elevated PTH levels  $>100$  pg/mL suggest secondary hyperparathyroidism and, in relation to the TRP, can discriminate between the underlying causes of hypophosphatemia. A high TRP with a low or normal PTH suggests phosphate deficiency, while a low TRP and high PTH suggests vitamin D or calcium deficiency [394].

MBDP tends to spontaneously resolve over time; however, it is necessary to supplement mineral postnatally to prevent the development of severe complications related to the bone disease and hypophosphatemia. It is considered appropriate to adopt preventative strategies of optimizing total parental nutrition and early achievement of full enteral feeding [380]. The essential preventative measures are to avoid postnatal risk factors, improve nutrition, and limit the use of chronic drugs that reduce mineral stores and enhance bone resorption. Adequate amounts of calcium and phosphate intake with TPN must be ensured and augmented during transition to enteral feeding and prolonged use of oral supplementation is further recommended during full enteral nutrition. The current practice recommendations when providing TPN in the first weeks of life are 40–120 mg/kg/day ( $1$ – $3$  mmol/L/kg/day) for calcium and 30–70 mg/kg/day ( $0.9$ – $2.2$  mmol/kg/day) for phosphate as shown in Table 63.3 [380,395]. At TPN fluid intakes of 150 mL/kg/day, 75–90 mg/kg/day ( $1.8$ – $2.2$  mmol/L/kg/day) for calcium and 60–70 mg/kg/day ( $1.9$ – $2.2$  mmol/kg/day) for phosphate are recommended with a ratio ranging from 1.5 to 1.7:1, to ensure a higher mineral accretion [395,396]. The recommended daily oral intake of calcium and phosphate also varies depending on the different consensus guidelines from the United States and European states outlined in the table [380,397,398]. The higher intake of protein

**TABLE 63.3** Treatment and supplementation of calcium, phosphate, and vitamin D in preterm infants receiving TPN and transitioned to enteral feeds,  $<1500$  g.

	TPN 1st few weeks	TPN after 1st few weeks (fluid intake 140–150 mL/kg/day)	Full enteral feeding
Calcium	40–120 mg/kg/day ( $1.0$ – $3.0$ mmol/kg/day)	75–90 mg/kg/day ( $1.8$ – $2.2$ mmol/kg/day)	140–160 mg/100 kcal (AAP) 70–140 mg/100 kcal (ESPGHAN)
Phosphate	31–71 mg/kg/day ( $1.0$ – $2.2$ mmol/kg/day)	60–70 mg/kg/day ( $1.9$ – $2.2$ mmol/kg/day)	95–108 mg/100 kcal (AAP) 50–86 mg/100 kcal (ESPGHAN)
Vitamin D	160–280 IU/day	160–280 IU/day	200–400 IU/day (AAP)

AAP, American Academy of Pediatrics; ESPGHAN, The European Society of Pediatric Gastroenterology Hepatology and Nutrition; TPN, total parenteral nutrition.

Reproduced with permission from Ref. [380].



and calories in TPN within the first few days of life helps increase the cellular uptake of phosphate.

Fortified human milk and preterm formulas meet the needs of the growing preterm infant as opposed to unfortified human breast milk, and these fortified milks are recommended until 36 weeks' gestation or at least until the infant reaches 2000 g. Phosphate supplementation should be commenced when serum phosphate level is  $<5.5$  mg/dL (1.3 mmol/L) to promote bone mineralization and prevent hypercalciuria [388]. Serum calcium levels are usually normal or marginally high due to elevated 1,25 dihydroxyvitamin D in response to hypophosphatemia. A calcium: phosphate ratio of 2:1 should be maintained to avoid hypercalciuria, nephrocalcinosis, and extraskeletal calcifications [394]. Calcium supplementation should be given if the TRP is reduced and PTH is elevated. The dose of vitamin D supplementation remains controversial in preterm infants as the different nutrition and pediatric societies recommend differing doses. It has been shown that 200–400 IU/day of vitamin D is sufficient to prevent osteopenia of prematurity and the AAP recommends 200–400 IU/day [388,399], although 1000 IU/day has sometimes been recommended in the literature. If the 25(OH)D levels are  $<30$  nmol/L, then higher doses of vitamin D may be warranted [397]. In the presence of concurrent liver or renal disease, 1,25 dihydroxyvitamin D (calcitriol) can be added to treat hypocalcemia [397]. If the infants sustain fractures that are often incidentally found, these fractures usually heal without orthopedic intervention unless they are severe and require skilled management (splinting, traction, or fixation) to promote better healing.

The chronic use of diuretics and steroids for bronchopulmonary dysplasia and caffeine that is commonly administered to treat apnea of prematurity are significant predisposing risk factors for MBDP. Both caffeine and furosemide have a diuretic effect and increase renal excretion of calcium. Caffeine increases osteoclastogenesis and causes hypercalcemia, thus reducing bone mineral content [400,401]. The exact mechanism of the action of steroids on bone in premature infants has not been studied but is likely to be due to both the direct and indirect effects of steroids on bone. The direct effects can occur rapidly with loss of bone mineral density due to increased bone resorption by increased expression of cytokines promoting osteoclastogenesis and thereafter chronically with a slower phase associated with decreased bone formation through the inhibition of osteoblast differentiation and the promotion of apoptosis [402,403]. Ali et al. studied 109 premature Canadian infants born  $<31$  weeks' gestation and  $<1500$  g who were admitted for 12 weeks in an intensive care unit and had radiological evidence of osteopenia of prematurity [378].

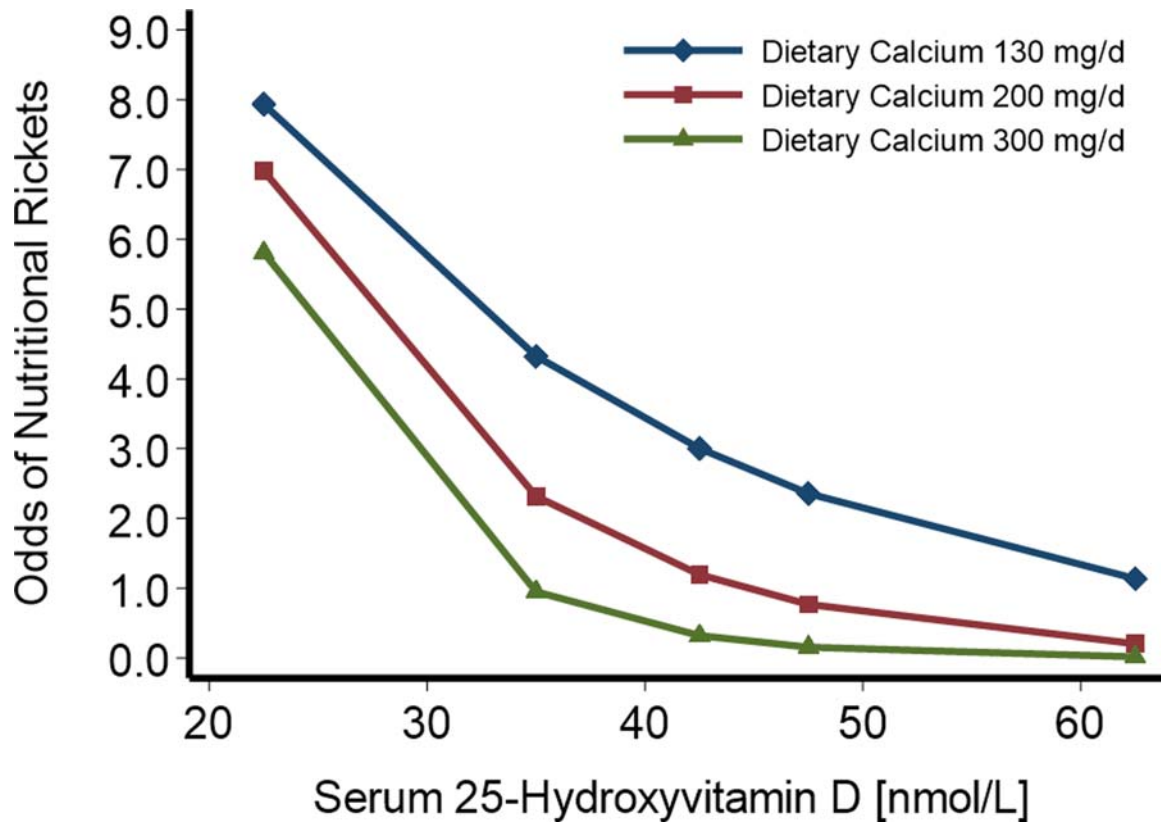
These infants had been receiving caffeine, steroids, diuretics, and vitamin D. There was a positive association of steroid cumulative dosages and the duration of caffeine use with osteopenia of prematurity in this study [378]. The chronic use of medication affecting bone mineral metabolism should be limited.

MBDP is generally a self-limiting condition in premature infants unless accompanied by severe osteopenia, underlying fractures and symptomatic biochemical abnormalities. Fig. 63.9 details an algorithmic approach to managing MBDP [397]. When a healthy preterm infant receives full enteral feeding with either fortified human milk or preterm formula within 2–3 weeks after birth, the infant should not be subjected to unnecessary investigations for MBDP.

### 13. The pathogenetic spectrum of nutritional rickets

Nutritional rickets has traditionally been viewed as being due to vitamin D deficiency through either an inadequate dietary intake or insufficient skin exposure to ultraviolet radiation, although more recently the role of a low dietary calcium intake in the face of a relatively normal vitamin D status has been considered to be an important pathogenetic mechanism in some LMICs. In addition to the classical causes of nutritional rickets, dietary intakes of calcium and phosphate are considered to play important roles in the pathogenesis of the metabolic bone disease of prematurity [397]. However, these pathogenetic concepts are too simplistic. Early studies by Mellanby [404] had shown the effect of cereals in exacerbating the clinical development of vitamin D deficiency rickets in dogs. Studies in baboons have confirmed these findings [348].

The resurgence of rickets and osteomalacia in the Asian community in Great Britain provided the impetus for detailed studies into the pathogenesis of vitamin D deficiency and bone disease in that community. Although vitamin D deficiency as assessed by circulating 25(OH)D concentrations is the hallmark of the disease in Asians [194,405,406], the mechanisms for the low vitamin D status and the high prevalence of rickets were unclear. It is apparent that the majority of Asians in Britain do not spend less time outdoors than their Caucasian counterparts [407]. Further, although they have darker skins than Caucasians, which might reduce the amount of vitamin D formed in response to sunlight exposure, Afro-Caribbean immigrants living in Britain have even darker skins, yet fewer cases of rickets have been described in this ethnic group [136]. Within the Asian community, studies have highlighted that risk factors for the disease include living at high latitude, Hindu



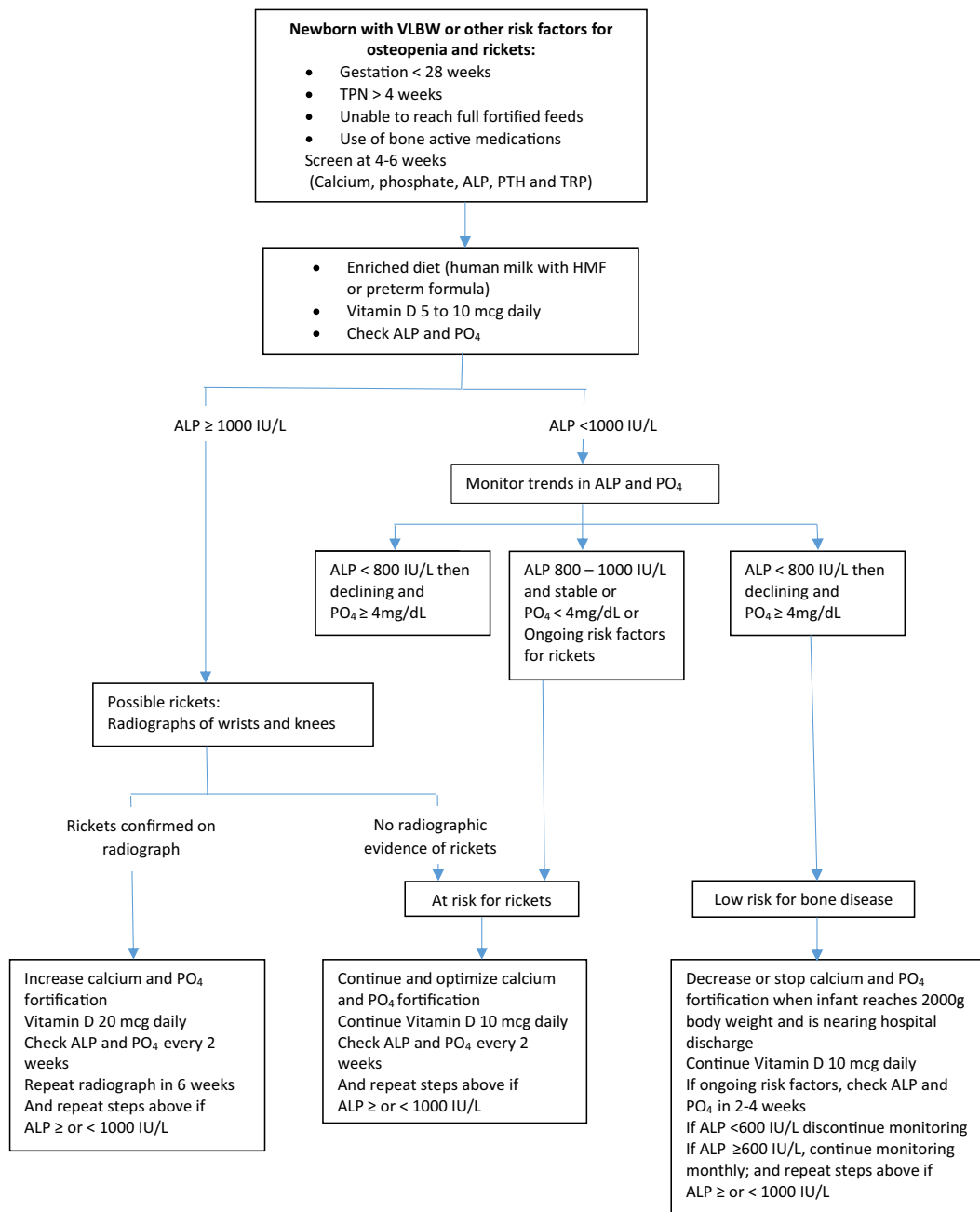
**FIGURE 63.8** The effect of changing 25-hydroxyvitamin D concentrations and dietary calcium intakes of the risk of developing rickets in Nigerian children. *Reproduced with permission from Ref. [137].*

religion, immigration from East Africa, vegetarianism, high-fiber diets, and the consumption of chapatti [134,135,408]. The association with vegetarianism, high-fiber diets, and cereals of high extraction suggests that dietary factors play a role. Support for this comes from two studies that have documented healing of rickets on removing chapattis from the diet [409,410], although this is not a universal finding [133].

Over the past four decades, research has shown that both high-fiber diets and intestinal malabsorption reduce the serum half-life of 25(OH)D by approximately one-third [411,412]. Further, experiments in rats have demonstrated that an elevation in serum 1,25(OH)<sub>2</sub>D concentrations, either by exogenous administration or endogenously through a low calcium diet, increases the metabolic clearance rate of 25(OH)D without altering its rate of production [232,413,414]. In these studies, the fall in serum 25(OH)D levels could be accounted for by an increase in polar metabolites appearing in the feces. Similar findings have been reported from studies in humans [233,415]. Conversely, increasing the calcium content of the diet has been shown to increase serum 25(OH)D and decrease serum 1,25(OH)<sub>2</sub>D concentrations [416]. Thus, these studies

convincingly show that dietary calcium and phytate content influences the catabolism of 25(OH)D through altering serum 1,25(OH)<sub>2</sub>D concentrations. In the face of a marginal vitamin D status due to limited skin synthesis of vitamin D and the low dietary vitamin D content of the diet, the increased catabolism is sufficient to precipitate vitamin D deficiency and clinical rickets and osteomalacia.

Thus, nutritional rickets has a spectrum of pathogenic mechanisms ranging from pure vitamin D deficiency associated with adequate calcium intakes, as might occur in the breastfed infant, at one end of the spectrum, to pure dietary calcium deficiency with an adequate vitamin D status, as documented in some Nigerian and South African rural children, at the other end [137,417] (Fig. 63.10). In between these two extremes lies the situation exemplified by the Asian community in Britain, where both poor calcium intakes or absorption and marginal vitamin D status combine to lead to frank rickets. It is likely that the high prevalence of rickets in vegetarian or immigrant children reported from the United States [56,57], Norway [418], Holland [419,420], and a number of tropical and subtropical countries [95] might be due to a mechanism similar to that in the Asian community,

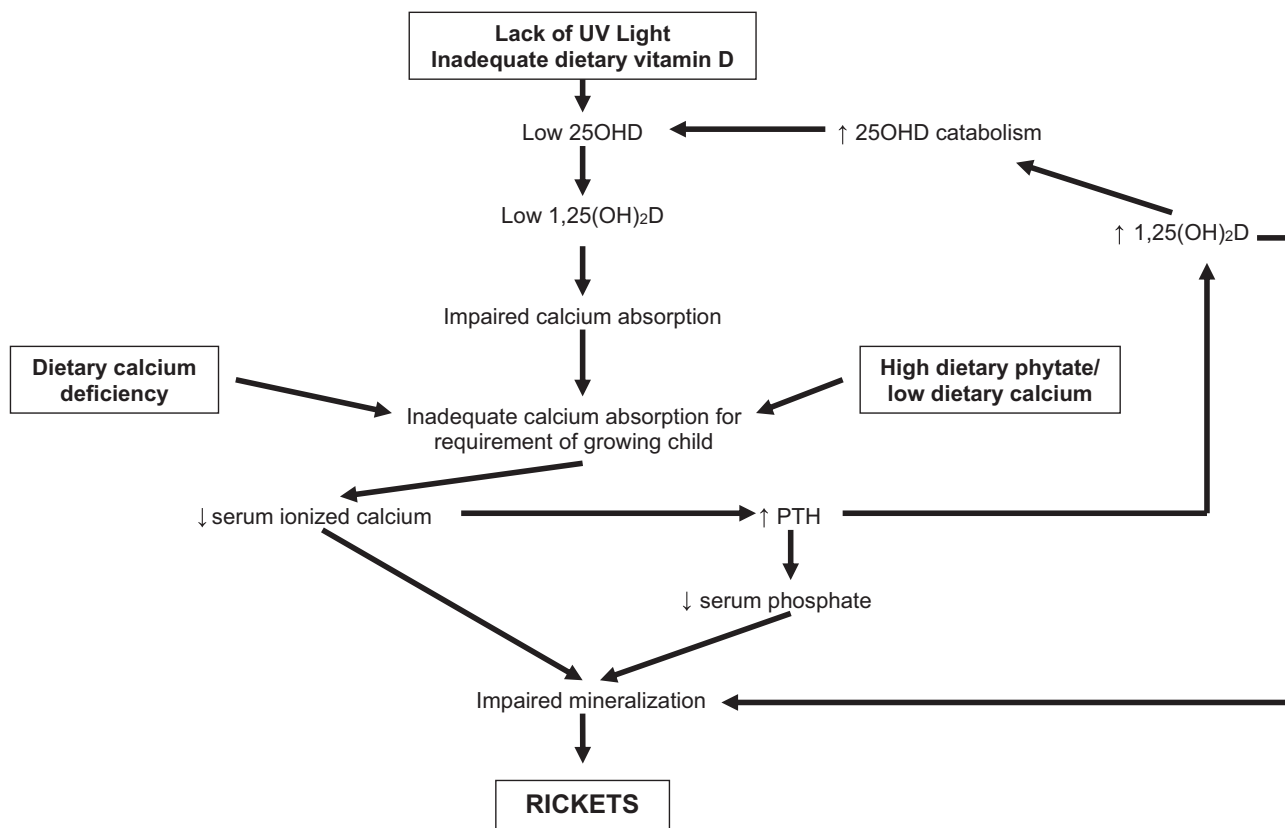


**FIGURE 63.9** An algorithmic approach to the diagnosis and management of the metabolic bone disease of prematurity. Adapted with permission from Ref. [397].

while osteomalacia in Bedouin adults in the Middle East reflects mainly dietary calcium deficiency [421].

A number of studies have highlighted the complex interaction between vitamin D and calcium intakes in the pathogenesis of nutritional rickets in children. A review of 43 patients diagnosed as having nutritional rickets in New Haven, Connecticut, found low 25(OH) D levels in only 22%, and the majority of infants had been weaned onto diets with minimal dairy content [117]. The authors concluded that low dietary calcium

intakes probably played a major role in the pathogenesis of the disease. Similar findings are reported from India, where it is suggested that low dietary calcium intakes were responsible for rickets in young children, while vitamin D deficiency played a major role in adolescents [317]. A recent reanalysis of data obtained from a large randomized controlled trial of the treatment of children with active nutritional rickets in Nigeria [275] has highlighted the synergistic effects of low dietary calcium intakes and a poor vitamin D status on the risk of



**FIGURE 63.10** The pathogenesis of nutritional rickets in children emphasizing the common final pathways of both dietary calcium deficiency and vitamin D deficiency resulting in hypocalcemia and hypophosphatemia and impaired mineralization.

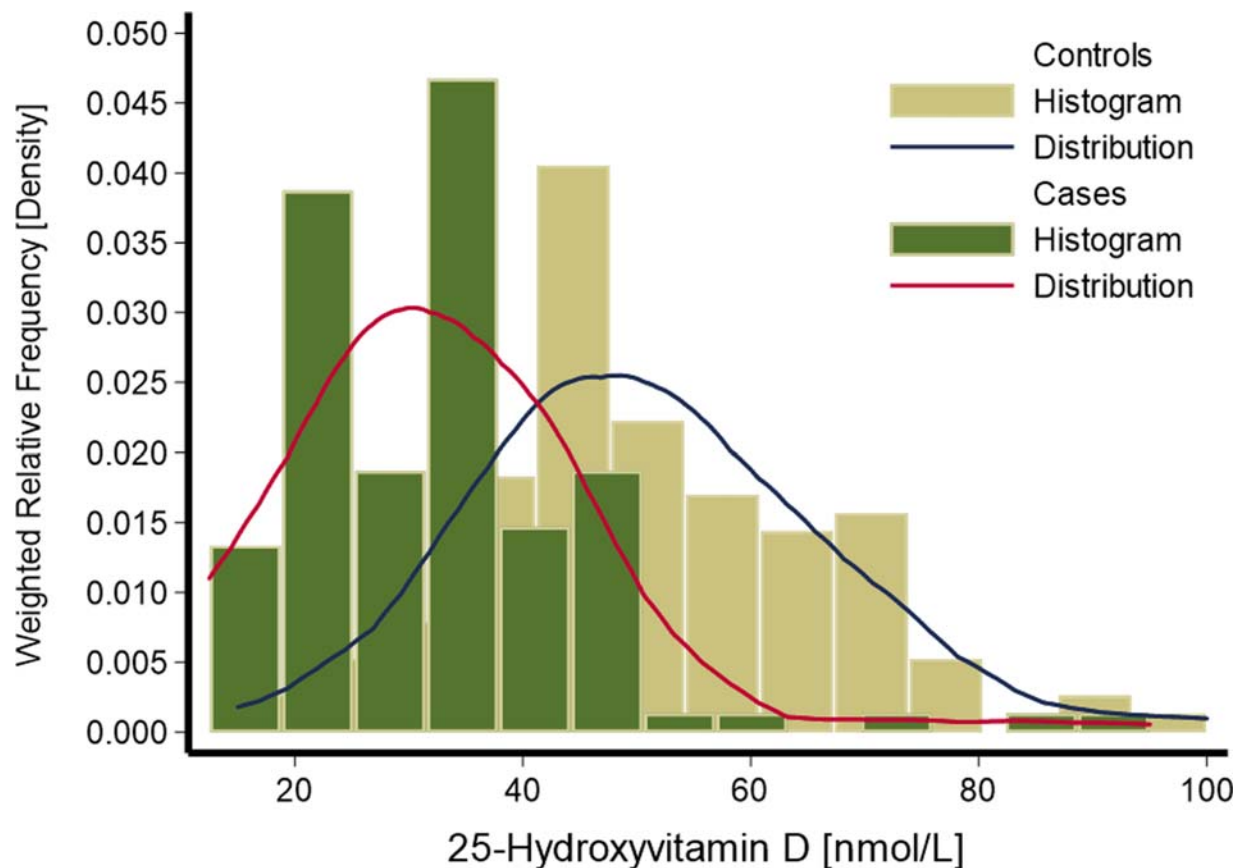
developing rickets in LMIC [137] (Figs. 63.8 and 63.11). Despite the clear evidence of the importance of these two factors in the pathogenesis of active rickets, the study was unable to tease out the factors that resulted in the poorer vitamin D status of affected children compared with that of community controls, or how some children were protected from developing rickets despite being on very low calcium intakes.

## 14. Conclusion

Despite readily accessible and effective means to eradicate nutritional rickets globally, the disease remains a major public health problem in many countries, not only in temperate regions of the world but also in tropical and subtropical countries. In many developed countries, the promotion of exclusive breastfeeding during the first 6 months of life and the concerns about the long-term effect of sunlight exposure during this period have exacerbated the risks of vitamin D deficiency in the young infant. In some subtropical countries, social customs play an important role in preventing adequate vitamin D status not only in the young

infant but also in the pregnant and lactating mother. In a number of developing countries, low dietary calcium intakes appear to play a major role in the pathogenesis of rickets in older children. Recent studies have helped to provide an all embracing concept of the interaction of vitamin D and calcium intakes in the pathogenesis of nutritional rickets. Further, the rapidly improving survival of very-low-birth-weight infants has highlighted the role of a lack of adequate dietary phosphorus intakes in the pathogenesis of metabolic bone disease of prematurity. In older infants and children, dietary phosphorus deficiency is a rare and unlikely cause of nutritional rickets due to the ubiquitous nature of phosphorus in foods; however, the special needs of the very-low-birth-weight infant have focused attention on metabolic bone disease of prematurity being a progressively important and preventable form on nutritional rickets.

There remains a need for international agencies to place the eradication of vitamin D deficiency and low calcium intakes among young children in many parts of the world as a priority. Nutritional rickets not only leads to an increased infant mortality but also has long-term sequelae.



**FIGURE 63.11** 25-Hydroxyvitamin D levels in Nigerian children with untreated nutritional rickets believed to be mainly due to dietary calcium deficiency and age- and sex-matched controls from the same community. Mean 25-hydroxyvitamin D is significantly lower in the rachitic subjects, but its concentration is above the generally recognized cut point for vitamin D deficiency of 30 nmol/L. *Reproduced with permission from Ref. [137].*

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# Clinical disorders of phosphate homeostasis

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## OBJECTIVES

- To describe the molecular and hormonal regulation of phosphate homeostasis.
- To describe the clinical presentation, genetic basis, and treatment of disorders of phosphate homeostasis, including those that present with hypophosphatemia or hyperphosphatemia.

## 1. Introduction

Extracellular phosphate is a critical component of the skeleton that plays an important role in modulating skeletal processes such as growth plate maturation and skeletal mineralization. Phosphate homeostasis is regulated by dietary intake and the regulators of mineral ion homeostasis, namely parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). Disorders disrupting phosphate homeostasis have resulted in a greater understanding of the molecular regulation of phosphate balance as well as the hormones that modulate phosphate homeostasis.

## 2. Phosphate homeostasis

Extracellular phosphate is an important component of the skeleton. Growing children have positive phosphate balance to support skeletal growth, while adults have a phosphate balance of zero. Osteomalacia in adults results if phosphate balance is negative. Phosphate accounts for

about 0.6% of the body weight at birth, and about 1% of body weight in the adult [1]. 85% of phosphate is in the skeleton and teeth, with the remaining 15% in the soft tissue and extracellular fluid [2].

In plasma, phosphate exists in either the inorganic or the organic form, which consists primarily of phospholipids and phosphate esters [3]. The total concentration of phosphate in plasma is about 14 mg/dL, of which 4 mg/dL is inorganic phosphate (Pi). Of phosphate, which is routinely measured in the clinical setting, namely Pi, 10%–15% is protein-bound and the remainder is filtered at the renal glomeruli and is usually complexed with sodium, calcium, or magnesium.

Sodium-dependent phosphate absorption in the small intestine is predominantly regulated by the sodium-dependent phosphate cotransporter type IIb (NPT2b, *SLC34A2*), with a minor contribution by the type III sodium-dependent phosphate cotransporters Pit1 and Pit2 [4]. Phosphate is then filtered by the kidney, where it is either reabsorbed or excreted depending on the requirements of the organism. About 60%–65% of dietary intake of phosphate is absorbed by the small intestine, where the higher the dietary load, the larger the amount of phosphate that is absorbed [1]. If an adult is in zero phosphate balance, the intestinal absorption of phosphate is equal to the amount of phosphate excreted by the kidney. In growing children, the net amount of phosphate excreted is less than the amount absorbed by the small intestine, thus resulting in positive phosphate balance [5]. Because serum phosphate concentrations vary throughout the day depending on dietary phosphate intake and oral phosphate supplementation, collection of a fasting blood sample is preferred for clinical analysis.

The proximal tubule of the kidney is the major site of phosphate reabsorption, with about 70% of the filtered load being reabsorbed in the proximal convoluted tubule, 10% in the proximal straight tubule, and <10% in the distal segments of the nephron [6]. Renal reabsorption of phosphate progressively increases as the filtered load increases, until a maximum tubular reabsorption rate for phosphate (TmP) is reached, after which phosphate excretion increases in proportion with the filtered load. The TmP can vary for the same person and between people due in part to the variation in GFR; therefore, TmP/GFR (maximum tubular reabsorption of phosphate per unit volume of GFR) is used as the quantitative estimate of renal tubular phosphate reabsorption [2].

Transport of phosphate across the renal tubule cells involves uptake of phosphate at the brush border apical membrane, translocation across the cell, and efflux at the basolateral membrane [7]. Renal reabsorption of phosphate is mediated by the brush border sodium-dependent ( $\text{Na}^+$ ) phosphate ( $\text{Pi}$ ) transporters, which depend on the  $\text{Na}^+/\text{K}^+$ -dependent ATPase on the basolateral membrane. There are three classes of  $\text{Na}/\text{Pi}$  cotransporters. Type I  $\text{Na}/\text{Pi}$  cotransporter (NPT1, *SLC17A1*) is expressed on the renal brush border membrane of the proximal tubule and can regulate the transport of  $\text{Pi}$  as well as other ions like chloride [8]. Interestingly, neither dietary phosphate nor PTH alters NPT1 protein or mRNA expression. Besides transporting phosphate, NPT1 also exports anions and urate.

Type II  $\text{Na}/\text{Pi}$  cotransporters include three highly homologous isoforms, including type IIa (NPT2a, *SLC34A1*), type IIc (NPT2c, *SLC24A3*), and type IIb (NPT2b, *SLC34A2*). NPT2a and NPT2c are exclusively expressed on the renal proximal tubule brush border membrane, while NPT2b is expressed in several tissues, including lung and the small intestine, but not the kidney [9–12]. NPT2a and NPT2c have eight membrane-spanning segments, where human NPT2a comprises 635 amino acids and its gene is located on chromosome 5q35, while human NPT2c comprises 599 amino acids and its gene is located on chromosome 9q34 [13]. In mice, Npt2c is approximately one order of magnitude less abundantly expressed than Npt2a [3].

Type III  $\text{Na}/\text{Pi}$  cotransporters are cell surface retroviral receptors (gibbon ape leukemia virus [Glv-1, Pit-1, *SLC20A1*] and murine amphotropic virus [Ram-1, Pit-2, *SLC20A2*]) that are ubiquitously expressed [14,15]. They are localized to all segments of the nephron at the basolateral membrane and mediate cellular phosphate balance.

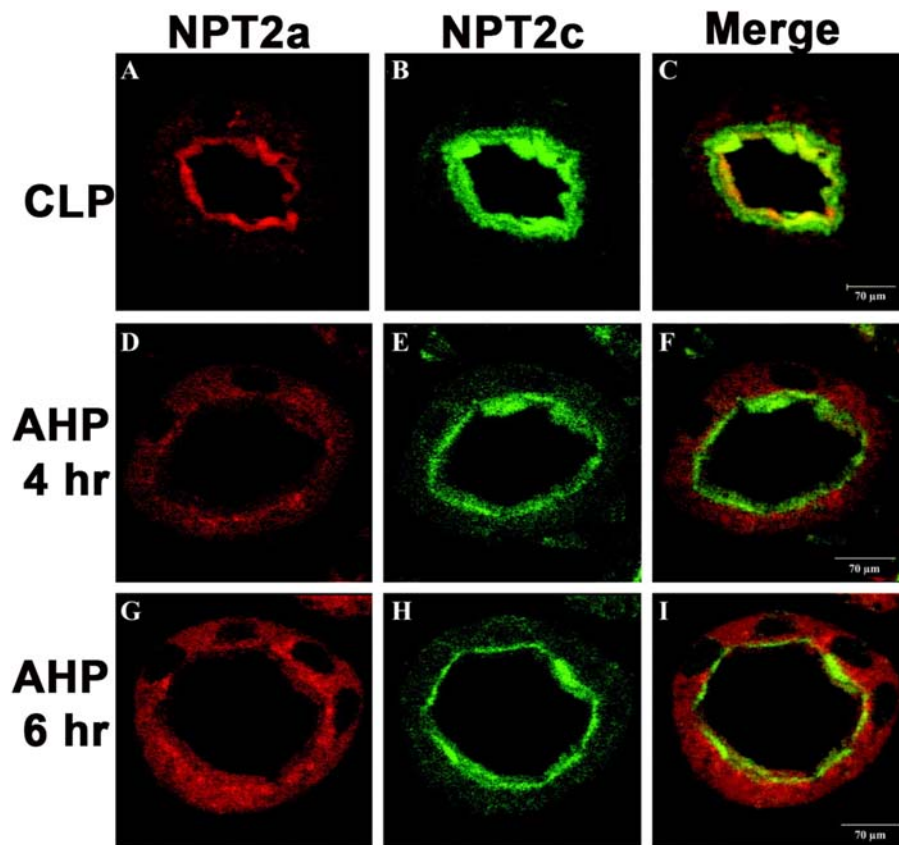
## 2.1 Regulation of phosphate homeostasis

Dietary intake as well as hormones, particularly parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23), play important roles in regulating phosphate

balance. Exposure to a low phosphate diet dramatically reduces urinary phosphate excretion [16], which was shown in rodents to be due to increasing Npt2a protein, but not NPT2a mRNA expression in the renal proximal tubule [17,18]. Subsequent studies demonstrated that low dietary phosphate does not activate NPT2a promoter activity [19]. In complementary studies, exposure to high phosphate diet results in the internalization of NPT2a protein and subsequent degradation by lysosomes via a microtubule-dependent process [20]. Likewise, a low phosphate diet increases NPT2c protein on the renal proximal tubular apical membrane, while NPT2c protein expression is decreased with exposure to high phosphate diet [21,22] (Fig. 64.1).

PTH regulates phosphate homeostasis by promoting the rapid internalization of NPT2a and its subsequent lysosomal degradation [23,24]. PTH binds to PTH/PTHrP receptors (PTHr1) on the basolateral membrane to increase cAMP signaling and to activate protein kinase A (PKA) or protein kinase C (PKC), while binding to receptors on the apical membrane is thought to activate only PKC [25]. NHERF2 interacts with the PTHr1 at the apical brush border membrane to coordinate the PTH-mediated activation of phospholipase C and inhibition of adenylyl cyclase through stimulation of inhibitory G proteins [26]. The PKA and PKC pathways may both activate ERK/MAPK signaling pathway to induce the PTH-mediated internalization of NPT2a [27,28]. Accessory proteins such as AKAP79 and RAP have been shown to play an important role in PTH-mediated NPT2a endocytosis [29,30]. Humans with NHERF1 mutations present with hypophosphatemia and renal phosphate wasting [31,32]; however, these findings are not universally observed [33]. It was demonstrated that NHERF1 stabilizes NPT2a on the membrane surface and can also prevent desensitization of the PTH signal [34].

A key regulator of phosphate balance is the hormone FGF23, which consists of 251 amino acid residues, including a signal peptide comprising 24 amino acids [35]. Referred to as a “phosphatonin,” FGF23 is a secreted FGF that modulates phosphate homeostasis. It was identified through genetic linkage studies to determine the cause of autosomal dominant hypophosphatemic rickets (ADHR), which resulted in the identification of mutations affecting either Arg176 or Arg179 that render FGF23 resistant to cleavage [36]. Especially in humans and mice on a low iron diet, the mutant FGF23 causes hypophosphatemia due to renal phosphate wasting [37,38]. FGF23 was also isolated from tumors that induce osteomalacia (TIO) because of abundant FGF23 expression [35] that is most likely triggered by expression of a chimeric FGFR/fibronectin protein [39]. Patients with this acquired disorder present with low serum phosphate levels and thus clinical



**FIGURE 64.1** Dietary phosphate regulates NPT2a and NPT2c: NPT2a and NPT2c are expressed on the brush border of the proximal renal tubule. Male Wistar rats were fed a low-phosphate diet for 2 days and then transitioned to one of three groups: chronic low-phosphate diet (CLP) or acute high-phosphate diet for 4 h (AHP 4 h) or 6 h (AHP 6 h). Immunohistochemical analysis of renal proximal tubular cells shows that the NPT2a and NPT2c cotransporters colocalize to the apical membrane (A–C) when rats are fed a low-phosphate diet. Administration of high-phosphate diet–induced translocation of NPT2a and NPT2c to intracellular compartments (D–I). *Figure modified from Ref. [21].*

symptoms that are similar to those observed in hypophosphatemic rickets. Administration of FGF23 to mice decreases serum phosphate and  $1,25(\text{OH})_2\text{D}$  levels, and it increases urinary phosphate losses [40,41]. Mice with vastly elevated FGF23 levels due to transplantation of cell lines stably expressing FGF23, or by expressing FGF23 transgenically under the control of different promoters, exhibit similar phenotypes with hypophosphatemia due to renal phosphate wasting, osteomalacia/rickets, and decreased  $1,25(\text{OH})_2\text{D}$  levels [42,43]. The opposite findings are encountered in FGF23-null mice that manifest hyperphosphatemia, increased serum  $1,25(\text{OH})_2\text{D}$  levels, and thus hypercalcemia [44,45]. FGF23 downregulates expression of *Cyp27b1*, the gene encoding the  $1\alpha$ -hydroxylase, and it upregulates *Cyp24a1*, the gene encoding an enzyme that metabolizes  $1,25(\text{OH})_2\text{D}$ , thus explaining the decreased circulating levels of  $1,25(\text{OH})_2\text{D}$  when FGF23 levels are elevated.

FGF23 undergoes O-linked glycosylation at Thr178 and probably other sites, which reduces cleavage at the RXXR site (residues 176–179) by a subtilisin-like proprotein convertase [46]. In contrast, cleavage is enhanced when

phosphorylation by FAM20C occurs at Ser180, which in turn reduces O-glycosylation at Thr178 [47]. Cleavage at the RXXR site generates a C-terminal 12 kDa fragment from the 32 kDa intact, glycosylated FGF23 [48]. FGF23 is expressed at low levels in a variety of tissues, including heart, liver, thymus, and brain [35,36]. However, it is predominantly expressed in bone, specifically osteocytes and at lower levels in osteoblasts [35,49]. Thus, osteoblast- or osteocyte-specific ablation of FGF23 results in a 40%–50% reduction in circulating FGF23 levels [50]. Loss-of-function variants in DMP1 [51], PHEX [52], FAM20C [53], and ENPP1 [54] lead to high circulating levels of FGF23 in mice and humans. Furthermore, it was recently shown that Glycerol-3-phosphate, a downstream product of glycolysis, is locally converted to lysophosphatidic acid (LPA), which stimulates FGF23 production. In vitro studies demonstrated that  $1,25(\text{OH})_2\text{D}$  in combination with LPA is required to increase FGF23 expression and that LPA increases VDR binding to the FGF23 promoter, suggesting that  $1,25(\text{OH})_2\text{D}$  action is downstream of LPA [55]. In the presence of the cofactor Klotho, FGF23 binds to FGFR1 with high affinity [56]. FGFR1 is the



predominant receptor for FGF23 action, with FGFR4 likely playing a minor role in the phosphaturic effect of FGF23 [57,58].

## 2.2 Phosphate and vitamin D metabolism

Dietary phosphate and serum phosphate concentration are important in the regulation of  $1,25(\text{OH})_2\text{D}$ . Acute hypophosphatemia induced in rats by dietary restriction increases the  $1\alpha$ -hydroxylase activity and thus the synthesis of  $1,25(\text{OH})_2\text{D}$  [59]. Likewise, dietary phosphate restriction in humans increases serum  $1,25(\text{OH})_2\text{D}$  levels, while phosphate supplementation decreases serum  $1,25(\text{OH})_2\text{D}$  concentrations [60]. Murine studies have furthermore demonstrated that dietary phosphate restriction increases CYP27B1 mRNA and the encoded  $1\alpha$ -hydroxylase protein in the renal proximal tubule by six- to eightfold increase [61]. Phosphate restriction furthermore decreases renal 24-hydroxylase activity and expression, thereby decreasing degradation of  $1,25(\text{OH})_2\text{D}$  [62,63]. Conversely, treatment of vitamin D-deficient rats with  $1,25(\text{OH})_2\text{D}$  enhances NPT2a mRNA expression and protein in the renal brush border membrane [64]. While the decrease in PTH that follows  $1,25(\text{OH})_2\text{D}$  administration could explain increased NPT2a levels, it has been reported that  $1,25(\text{OH})_2\text{D}$  can directly enhance NPT2a promoter activity and protein level in the kidney [64].

## 2.3 Clinical symptoms of hypophosphatemia and hyperphosphatemia

Moderate to severe hypophosphatemia can be associated with clinical symptoms [65]. Although chronic hypophosphatemia is usually asymptomatic, acute severe hypophosphatemia concurrent with phosphate depletion can lead to significant clinical manifestations. Low serum phosphate is commonly a consequence of chronic alcoholism [66,67]. A combination of other factors, including inappropriate phosphaturia, gastrointestinal losses, hyperventilation, or infusion of dextrose-containing fluids, can contribute to the hypophosphatemia in alcoholism [65].

Hematologic consequences of hypophosphatemia may include hemolysis or leukocyte dysfunction. Adequate phosphate levels are necessary to maintain ATP levels in red blood cells and leukocytes [68]. The fall in ATP levels from hypophosphatemia has been associated with decreased life span of red blood cells and can cause hemolytic anemia [69–71]. It has been reported that decreased ATP levels in leukocytes in hypophosphatemic patients can impair cell function, notably chemotaxis and phagocytosis [72,73].

Compromised respiratory function can also be due to hypophosphatemia. Respiratory muscle weakness,

including impaired diaphragm function, can occur with low phosphate levels [74,75]. In hospitalized patients who had low phosphate levels, improvement in respiratory muscle function was observed with phosphate repletion. A decrease in myocardial function has also been noted with severe acute hypophosphatemia [76,77]. However, a study of pediatric patients with chronic hypophosphatemia from X-linked hypophosphatemia (XLH) demonstrated no alteration in left ventricular function [78]. Central and peripheral nervous system dysfunction has been reported in patients with acute severe hypophosphatemia after developing refeeding syndrome [79,80]. Patients may manifest with irritability and muscle weakness and progress to confusion or coma [80,81].

Hyperphosphatemia is generally asymptomatic. A significant consequence of elevated phosphate levels is hypocalcemia due to the precipitation of calcium-phosphate deposits in soft tissue. These ectopic calcifications have been observed in renal failure, hypoparathyroidism, pseudohypoparathyroidism, or tumoral calcinosis, all of which are disorders associated with hyperphosphatemia. Biochemical consequences of elevated phosphate levels can be secondary hyperparathyroidism or the suppression of production of  $1,25(\text{OH})_2\text{D}$ .

## 3. Disorders of phosphate homeostasis

There are many disorders that affect phosphate homeostasis, including inherited syndromes as well as acquired disorders (Table 64.1). In recent years, the genetic cause for many of these diseases has been identified (Fig. 64.2). The aberrant genes are often lead to abnormalities in FGF23 function or phosphate transport.

### 3.1 Fanconi syndrome

In Fanconi syndrome, disruption to the function of the proximal renal tubule by genetic or secondary causes results in hypophosphatemia, proximal renal tubular acidosis, and renal losses of amino acids, glucose, bicarbonate, and phosphate. The hypophosphatemia was thought to be a consequence of inappropriately low levels of serum  $1,25(\text{OH})_2\text{D}$  (Fanconi syndrome, type 1) [82,83]. Affected individuals can present with clinical findings consistent with osteomalacia and rickets, with bowing of the legs and short stature. Histomorphometric analyses show decreased cancellous bone volume and increased osteoid [83]. Treatment involves supplementation with phosphate and/or calcitriol. Rare inherited disorders, which may be complicated by Fanconi syndrome, include cystinosis, Wilson's disease, Lowe syndrome, type I glycogen storage disease, or galactosemia. The majority of cases have an acquired form of Fanconi syndrome due to secondary causes

**TABLE 64.1** Disorders of phosphate homeostasis.**Hypophosphatemic syndromes**

Fanconi's syndrome

*Genetic diseases with excess FGF23*

X-linked hypophosphatemia (XLH)

Autosomal dominant hypophosphatemic rickets (ADHR)

Autosomal recessive hypophosphatemic rickets (ARHR)

Fibrous dysplasia/McCune Albright syndrome

Linear nevus sebaceous syndrome (LNSS)/epidermal nevus syndrome (ENS)

*Acquired diseases with excess FGF23*

Tumor induced osteomalacia (TIO)

Chronic kidney disease

*Diseases with low FGF23*

Mutations in NPT2a (infantile hypercalcemia)

Mutations in NPT2c (hereditary hypophosphatemic rickets with hypercalciuria)

**Hyperphosphatemic syndromes**

Tumoral calcinosis

**Disorders of altered phosphate load**

Phosphate deprivation

Gastrointestinal malabsorption

Alkalosis

Glucose administration

Alcoholism

Burns

Diabetic ketoacidosis

Refeeding syndrome

that may include burn injuries, exposure to certain medications or toxic metals, or a variety of other conditions such as multiple myeloma or amyloidosis [83]. Tenofovir, an anti-retroviral medication used for treatment of HIV/AIDS, is well documented to be associated with hypophosphatemia due to development of Fanconi syndrome [84]. Patients infected with acute respiratory syndrome coronavirus (SARS-CoV-2), who have severe disease requiring hospitalization, may have a higher incidence of hypophosphatemia associated with acute kidney injury. It is hypothesized SARS-CoV-2 infection of the proximal renal tubules may lead to Fanconi syndrome [85].

An autosomal recessive form of renal Fanconi syndrome was described in 1988 in two siblings that were products of a consanguineous marriage. Both had

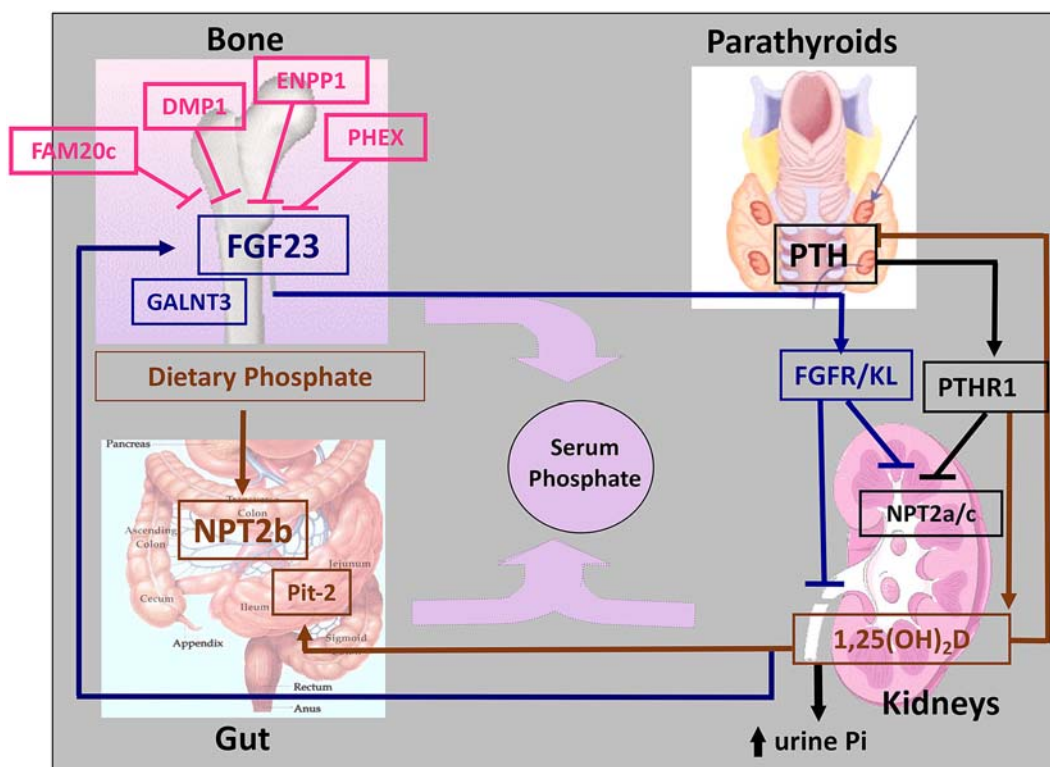
marked hypophosphatemia and elevated 1,25(OH)<sub>2</sub>D levels, associated with rickets, growth retardation, and skeletal deformities [86]. At the ages of 39 and 43 years, when a homozygous loss-of-function mutation was found in *SLC34A1* that encodes NPT2a, both siblings had only mild hypophosphatemia, normal 1,25(OH)<sub>2</sub>D levels, and no increase in urinary calcium excretion, yet had impaired renal function [87]. Recently, numerous other homozygous or compound heterozygous NPT2a mutations were described in several infants with severe hypophosphatemia, decreased TmP/GFR, as well as severe hypercalcemia and hypercalciuria caused by elevated 1,25(OH)<sub>2</sub>D levels [88].

### 3.2 Genetic hypophosphatemic syndromes of excess FGF23

#### 3.2.1 Hypophosphatemic rickets—X-linked hypophosphatemia

X-linked hypophosphatemia (XLH) is the most frequent inherited form of hypophosphatemic rickets, where the incidence is reported at 1:20,000 [89]. It is inherited in an X-linked dominant pattern with complete penetrance, where males and females are affected equally. The phenotype can vary widely even in the same family. Diagnosis of XLH can be delayed until adulthood due to lack of awareness of phosphate levels or expression of a mild phenotype. Affected individuals often present with rickets and osteomalacia, causing short stature and bowed legs. The growth plates are abnormally expanded; therefore, the metaphyses of long bones are widened [90]. There is discordant growth between the long bones and the torso, with the growth retardation being more apparent in the long bones [91]. Clinical presentation of XLH may also include dental abscesses due to defective dentin or enamel, and cranial synostosis [92,93]. Adults may manifest with pseudo-fractures, osteoarthritis, osteophytes, or enthesopathy, which is the abnormal mineralization of the bony insertion sites of tendons and ligaments [94,95]. Patients may also report muscle weakness or cramping, though many XLH patients are often asymptomatic from the hypophosphatemia [2]. As adults, XLH patients also report tinnitus and conductive hearing loss. Mice with XLH similarly have conductive hearing loss, which is associated with hypomineralization of the auditory ossicles [96].

Most individuals affected by XLH have elevated (or inappropriately normal) serum FGF23 levels, thus explaining their hypophosphatemia and renal phosphate wasting, as well as their low or inappropriately normal 1,25(OH)<sub>2</sub>D levels caused by suppression of the renal 1 $\alpha$ -hydroxylase [49]. Additional biochemical characteristics of XLH include normal serum calcium



**FIGURE 64.2** Molecular pathways underlying genetic disorders of phosphate homeostasis: Dietary phosphate is absorbed in the gut by NPT2b and filtered by the kidney. In the small intestine, phosphate transport is predominantly regulated by NPT2b. 1,25(OH)<sub>2</sub>D increases Pit-2 expression in the small intestine, which also enhances phosphate absorption, and feeds back to decrease PTH production by the parathyroid glands. Pit-1 is expressed in the small intestine; however, a role for this transporter in phosphate absorption has not yet been defined. PTH binds to the PTHR1 to reduce NPT2a and NPT2c protein levels, resulting increased renal loss of phosphate. FGF23 is predominantly expressed in bone and binds to the FGFR with  $\alpha$ -Klotho (KL) as coreceptor in the kidneys. It thus increases, like PTH, urine phosphate excretion by decreasing renal proximal tubule expression of NPT2a and NPT2c. FGF23 furthermore stimulates 24-hydroxylase expression, and it reduces the 1- $\alpha$ -hydroxylase. The combined net effects of the actions of FGF23 in proximal renal tubules are decreased serum levels of phosphate and 1,25(OH)<sub>2</sub>D. FGF23 expression is down-regulated by PHEX, DMP1, ENPP1, and FAM20C. Heterozygous loss-of-function mutations in PHEX cause X-linked hypophosphatemia (XLH), while homozygous loss-of-function mutations in DMP1, ENPP1, or FAM20C lead to autosomal recessive hypophosphatemic rickets (ARHR). Mutations at the cleavage site of FGF23 increase circulating intact FGF23 and therefore cause autosomal dominant hypophosphatemic rickets (ADHR), especially in the presence of iron deficiency. The different forms of hyperphosphatemic familial tumoral calcinosis are caused by homozygous mutations in GALNT3, FGF23, or  $\alpha$ -Klotho. GALNT3 mediates the glycosylation and stabilization of FGF23, while  $\alpha$ -Klotho is the coreceptor required for FGF23 action in the kidney. Homozygous or compound heterozygous mutations in NPT2a lead to renal Fanconi syndrome or a more severe phosphate-wasting disorder associated with hypercalcemia, previously referred to as idiopathic infantile hypercalcemia. Homozygous or compound heterozygous mutations in NPT2c cause hereditary hypophosphatemic rickets with hypercalciuria (HHRH).

and 25(OH)D levels, and serum PTH levels that are frequently elevated even before initiation of oral phosphate supplements [97,98] (Table 64.2).

A murine equivalent of human XLH is the Hyp mouse, which has a deletion in the 3' region of the PHEX gene; [99] PHEX mutations have also been identified in other mice with XLH, including Gy [100], Ska1 [101], Hyp-Duk, Hyp-2J [102], Pug [103], and Kbus/Idr [104] mouse models. The Hyp mouse has a phenotype that is very similar to that of humans with XLH, namely hypophosphatemic rickets due to renal phosphate wasting, low serum phosphate and 1,25(OH)<sub>2</sub>D levels, and consistently elevated serum FGF23. As a result of the increased FGF23 action, expression of 1 $\alpha$ -hydroxylase is reduced, while 24-hydroxylase expression is

increased, thus leading through two mechanisms to a decrease in circulating 1,25(OH)<sub>2</sub>D levels [49].

### 3.2.1.1 Pathophysiology of XLH

XLH is caused by inactivating mutations in the PHEX gene, which is located on chromosome Xp22.1 [105]. A large variety of different mutations throughout the PHEX gene have been described, including missense, frameshift, nonsense, duplication, and insertion mutations [106,107]. A database of mutations identified in PHEX can be found at the website [www.db.mcgill.ca](http://www.db.mcgill.ca). PHEX is expressed in several tissues such as lung, brain, and teeth, but it is predominantly expressed in bone, specifically in osteocytes and osteoblast cell types [52,108]. The PHEX protein shows significant homology

**TABLE 64.2** Biochemical findings in disorders of phosphate homeostasis.

	XLH	ADHR	ARHR	TIO	Infantile hypercalcemia	HHRH	TC
Serum Pi	↓	↓	↓	↓	↓	↓	↑
TmP/GFR	↓	↓	↓	↓	↓	↓	↑
FGF23	↑/N	↑/N	↑/N	↑/N	↓/N	↓/N	Intact: ↓ C-term: ↑
Serum Ca	N	N	N	N	N/↑	N/↑	N
Urine Ca	N	N	N	N	↑	↑	N
PTH	N/↑	N/↑	N/↑	N	↓	↓	↓
25(OH)D	N	N	N	N	N	N	N
1,25(OH) <sub>2</sub> D	↓/N	↓/N	↓/N	↓/N	↑/N	↑/N	↑/N
Mutant gene	PHEX	FGF23	DMP1, ENPP1, FAM20C		NPT2a	NPT2c	FGF23, GALNT3, KLOTHO

ADHR, autosomal dominant hypophosphatemic rickets; ARHR, autosomal recessive hypophosphatemic rickets; HHRH, hereditary hypophosphatemic rickets with hypercalciuria; N, normal; TC, tumoral calcinosis; TIO, tumor-induced osteomalacia; XLH, X-linked hypophosphatemia; ↓, decreased, ↑, increased.

to the M13 family of zinc metallopeptidases, which include neutral endopeptidase neprilysin (NEP), endothelin-converting enzyme 1 (ECE-1 and 2), and the Kell antigen [108,109]. This group of proteins are type II integral membrane glycoproteins that have a short N-terminal cytoplasmic domain, a single transmembrane hydrophobic region, and a large extracellular domain. The direct target of PHEX endopeptidase activity currently remains unknown. It was initially thought that PHEX cleaves FGF23 [110]. However, it has since been determined that while FGF23 is overexpressed in the setting of PHEX mutations, its cleavage is not altered by these mutations [110,111]. Bones in Hyp mice have increased expression of MEPE (matrix extracellular phosphoglycoprotein), which contains an acidic, serine, and aspartic acid-rich motif (ASARM). Studies demonstrated that PHEX binds to and cleaves the triphosphorylated ASARM motif in proteins like MEPE to block their inhibition of mineralization [112].

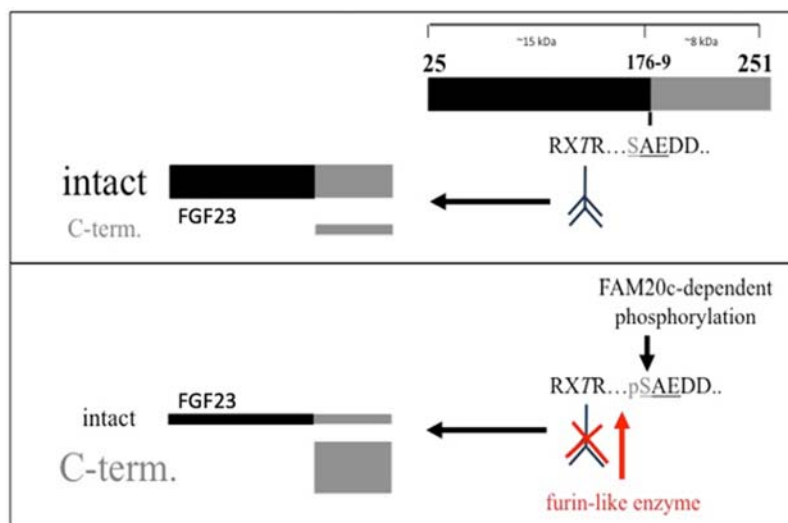
Investigations into the pathogenesis of XLH have confirmed the roles of PHEX and FGF23 in developing the rachitic phenotype. Conditional ablation of PHEX in osteoblasts (using the osteocalcin promoter) resulted in osteomalacia with elevated serum FGF23 levels, hypophosphatemia, and decreased renal Npt2a expression [52]. However, while overexpression of PHEX under the control of the pro- $\alpha$ 1(I) collagen promoter in Hyp mice improved the skeletal mineralization, it did not normalize serum phosphate levels [113]. Moreover, overexpression of FGF23 under the  $\alpha$ 1(I) collagen promoter recapitulated the hypophosphatemic rickets phenotype, including decreased renal expression of Npt2a, hypophosphatemia, and phosphaturia, and a decrease in skeletal mineralization with growth retardation [114]. Deletion of FGF23 from Hyp mice reversed the low serum phosphate and 1,25(OH)<sub>2</sub>D levels, but

did not normalize skeletal mineralization or growth, supporting a role for FGF23-specific actions on these processes [49]. A few studies have provided in vitro evidence that FGF23 may suppress mineralization independent of its effect on phosphate homeostasis. Overexpression of FGF23 in rat calvarial osteoblasts resulted in decreased mineralization and nodule formation, while there was decreased bone formation in parietal bone organ culture [115]. Treatment of wild-type calvarial osteoblasts furthermore suppressed formation of mineralized nodules in vitro [116].

The defect in skeletal mineralization in the XLH phenotype may not be attributable to just the mineral ion and hormone abnormalities. There is an accumulation of the mineralization inhibitor osteopontin, a target of PHEX, in the extracellular matrix of Hyp bone. Deletion of osteopontin in the Hyp mice modestly improved skeletal mineralization [117]. Furthermore, implantation of Hyp osteoblasts into wild-type mice reduced, but did not normalize the osteoid thickness and volume of the transplants, thus suggesting an intrinsic osteoblast defect in Hyp mice [118]. When wild-type osteoblasts were grown in medium with phosphate concentrations similar to those of serum levels in wild-type mice, alkaline phosphatase activity increased. In contrast, Hyp osteoblasts failed to respond to 1,25(OH)<sub>2</sub>D treatment when exposed to the same phosphate concentration, thus demonstrating that Hyp osteoblasts have an abnormal response to 1,25(OH)<sub>2</sub>D [119].

Studies have furthermore demonstrated the critical importance of extracellular phosphate for normal growth plate maturation [120–122]. The murine model of XLH (Hyp mouse) shows an expansion of growth plates that is similar to that of mice with ablation of the vitamin D receptor and mice fed a low phosphate diet. These mice all have hypophosphatemia and





**FIGURE 64.3** Fam20C phosphorylates FGF23: FAM20C is a kinase located in the endoplasmic reticulum/Golgi network that phosphorylates proteins with the SXE motif. FGF23 is phosphorylated by FAM20C on S180, which is adjacent to the 176-RXXR-179 SPC cleavage site. Phosphorylation of FGF23 on S180 (shown as pS) reduces glycosylation of the T178 by GALNT3 (red cross), therefore rendering FGF23 more susceptible to cleavage by a furin-like enzyme (red arrow), resulting in the generation of increased levels of C-terminal FGF23 fragments. Conversely, homozygous Fam20C mutations prevent phosphorylation at S180, thus enhancing glycosylation and stability of FGF23, consequently promoting hypophosphatemia. *Modified from Ref. [47].*

growth plate expansion, along with a decrease in apoptotic hypertrophic chondrocytes [120]. Erk1/2 activation, which is necessary for hypertrophic chondrocyte cell death, is decreased in the growth plate [122]. PTHrP action is also thought to be necessary for the growth plate expansion observed in low phosphate conditions: mice heterozygous for the ablation of PTHrP did not develop the anticipated growth plate expansion when fed a low phosphate diet [123]. Since mice and humans with XLH have elevated serum PTH levels and Hyp mice have increased expression of PTHrP at the periaricular surface, the actions of both ligands may contribute to the abnormal growth phenotype.

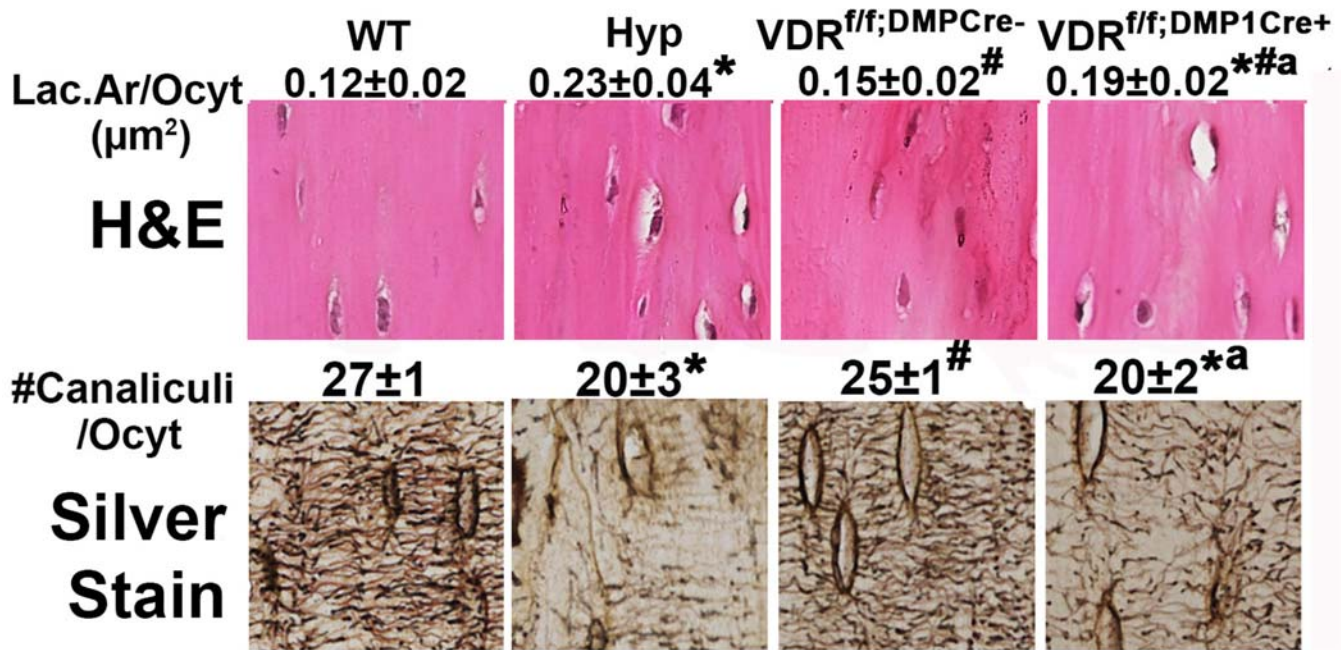
In addition to its ability to increase serum calcium and phosphate levels, 1,25(OH)<sub>2</sub>D is also important for growth and skeletal mineralization and structure in XLH. The excess FGF23 in XLH suppresses 1 $\alpha$ -hydroxylase and increases vitamin D 24-hydroxylase expression to decrease levels of 1,25(OH)<sub>2</sub>D. Ablation of vitamin D 24-hydroxylase (*Cyp24a1*) in Hyp mice or mice overexpressing FGF23 resulted in significant amelioration of the osteomalacia and rachitic changes observed with XLH despite persistent hypophosphatemia [124]. Although serum 1,25(OH)<sub>2</sub>D levels decreased, there was an increase in local tissue production of 1,25(OH)<sub>2</sub>D [124]. In addition, treatment of Hyp mice with calcitriol alone was sufficient to improve growth, growth plate maturation, skeletal microarchitecture, and dentoalveolar mineralization; in fact, growth was improved more significantly with 1,25(OH)<sub>2</sub>D than treatment with the anti-FGF23 blocking antibody despite increased serum levels of FGF23

[89,125]. Administration of calcitriol alone also restored Npt2a expression in the kidney [89] (Fig. 64.3).

Hyp cortical bone is characterized by dramatically impaired osteocyte lacunocanicular organization with enlarged lacunae and sparse canaliculi. Treatment of Hyp mice with either calcitriol alone or the anti-FGF23 antibody normalizes osteocyte lacunar size and partially restores canicular structure [126]. Deletion of the vitamin D receptor (VDR) in osteocytes similarly results in enlarged osteocyte lacunae and impaired canicular structure, thus suggesting that impaired 1,25(OH)<sub>2</sub>D signaling in Hyp mice contributes to the osteocyte lacunocanicular abnormalities (Fig. 64.4) [127]. While the pathophysiology underlying the paradoxical mineralization of the bone–tendon attachment sites, called enthesopathy, observed in XLH is poorly understood, recent studies demonstrate that cells in the Hyp entheses have increased alkaline phosphatase activity and enhanced bone morphogenic protein (BMP) and Indian hedgehog (IHH) signaling, both of which regulate chondrogenesis. Treatment of Hyp mice with either calcitriol alone or the anti-FGF23 blocking antibody prevents enthesopathy in Hyp mice, also pointing to a role for impaired 1,25(OH)<sub>2</sub>D action in XLH enthesopathy [128]. Taken together, these studies support an important role for 1,25(OH)<sub>2</sub>D in the pathogenesis of the skeletal phenotype observed in XLH.

### 3.2.1.2 Treatment of XLH

Treatment for XLH in children is predominantly aimed at improving their growth, to minimize rickets/



**FIGURE 64.4** Decreased 1,25(OH)<sub>2</sub>D action contributes to the impaired lacunocanalicular structure observed in bones from mice with XLH (Hyp): H&E and silver stain of day 30 tibia in Hyp mice and mice lacking the vitamin D receptor (VDR) in osteocytes (VDR<sup>f/f</sup>;DMP1<sup>Cre+</sup>), with wild-type (WT) and VDR<sup>f/f</sup>;DMP1<sup>Cre-</sup> controls. The lacunae are visualized by H&E stain, and lacunar area per osteocyte (Lac.Ar/ocyt) is quantitated by histomorphometry. The canalicular network is visualized by silver stain and canalicular density (#canaliculi/ocyt) quantitated. Both Hyp and VDR<sup>f/f</sup>;DMP1<sup>Cre+</sup> tibiae have enlarged lacunae and decreased canalicular density compared with controls, indicating that an impaired production of 1,25(OH)<sub>2</sub>D contributes to the osteocyte lacunocanalicular network abnormalities observed in Hyp mice. \* =  $P < .05$  versus WT, # =  $P < .05$  versus Hyp, a =  $P < .05$  versus VDR<sup>f/f</sup>;DMP1<sup>Cre-</sup>. Figure modified from Ref. [127].

osteomalacia, and thus to prevent bowing. Children with XLH are often treated with conventional therapy with a combination of oral phosphate, given multiple times per day, and calcitriol, from the time of diagnosis until active growth has stopped. However, these pediatric patients are increasingly being treated with a humanized antibody targeting FGF23, named burosumab (Crysvita), which is FDA approved for the treatment of children and adults with XLH. Administration of a combination phosphate and calcitriol therapy can decrease alkaline phosphatase levels and improve, but not normalize skeletal growth and mineralization [90,97]. Serum phosphate levels do not normalize with conventional therapy because of persistent renal phosphate losses due to elevated circulating FGF23 levels. In fact, treatment of XLH patients with this combination therapy further increases serum FGF23 levels [129]. In children whose growth retardation is refractory to standard therapy, adjuvant therapy with growth hormone has improved growth velocity in some studies [130,131]. However, outcomes with growth hormone therapy are variable since some studies reported only transient or no improvement in biochemical parameters, lack of improved growth velocity, and potential worsening of skeletal complications [91,131–133]. Unlike children, most adults with XLH are only treated if they have active osteomalacia, are

undergoing orthopedic procedures, or have symptoms such as bone pain or muscle cramps and fatigue.

Complications of conventional therapy may include hypercalciuria or hypercalcemia from calcitriol therapy [134]. Also, the doses of phosphate (and compliance with this medication) and calcitriol are thought to be correlated with the development of nephrocalcinosis, which can lead to compromised renal function [135]. Phosphate therapy alone leads to secondary and tertiary hyperparathyroidism, requiring parathyroid surgery [136]. Therefore, calcitriol is normally added to suppress PTH synthesis and secretion. In addition, calcimimetics such as cinacalcet have been reported to decrease PTH levels in XLH patients, thus decreasing the PTH-dependent urinary phosphate losses [137]. Because of the toxicities associated with standard therapy, close monitoring of biochemical parameters in actively treated XLH is needed. It is also unclear if conventional treatment with oral phosphate and low doses of calcitriol therapy can prevent or treat complications of XLH such as enthesopathy, osteoarthritis, or dental abscesses. In an observational cross-sectional study, therapy with combination calcitriol (1,25(OH)<sub>2</sub>D) and oral phosphate in adults with XLH did not alter the number of entheses sites affected, but may be associated with a lower risk for dental complications [92]. Treatment of mice with XLH

with calcitriol starting prior to the development of enthesitis can prevent enthesopathy [128], but treating Hyp mice with calcitriol and phosphate starting at day 30 (after enthesitis maturation) did not prevent or reduce the extent of enthesopathies [138], suggesting early and consistent therapy is needed to attenuate enthesopathy.

Due to the lack of normalization of the XLH phenotype with conventional therapy, a novel antibody targeting FGF23, burosumab, was developed. The antibody has been studied in adults with XLH, demonstrating that antibody treatment for up to 16 months resulted in significant and sustained increases in serum phosphate and TmP/GFR, while serum 1,25(OH)<sub>2</sub>D levels were transiently increased and normocalcemia was maintained [139,140]. By 48 weeks of therapy, treated adults did not develop hyperparathyroidism, hypercalciuria, or worsening nephrocalcinosis. Adults on burosumab had significant improvement in fracture/pseudofracture healing, skeletal mineralization, and reported pain score, though no decrease in stiffness or improved physical function, as compared with adults treated with placebo [141–143]. In pediatric subjects aged 1 to 12 with severe rickets, burosumab was superior to conventional therapy in improving Rickets Severity Score, serum mineral ion and hormone levels, and growth, although the increase in growth z score was rather modest at 64 weeks [144]. Similar to children with XLH, adults treated with burosumab also demonstrated consistent normalization serum phosphate levels to the low-normal range. Given the efficacy of burosumab in improving serum biochemistries, growth, and skeletal mineralization, it will likely replace conventional therapy as the treatment of choice for XLH. Both treated children and adults continued to develop dental abscesses, and the effects of burosumab on the development of enthesopathies or osteoarthritis have not been reported. Murine studies comparing an alternate FGF23 blocking antibody with daily calcitriol therapy (without phosphate supplementation) in mice with XLH revealed that daily calcitriol was superior to the antibody in improving growth plate morphology, long bone growth, histomorphometric parameters, and skeletal mineralization and biomechanics [89]. Thus, calcitriol alone or in combination with burosumab may potentially be alternative treatment options for XLH patients.

### 3.2.2 Autosomal dominant hypophosphatemic rickets

ADHR is an even rarer disorder characterized by laboratory and clinical findings similar to XLH (Table 64.2). Penetrance is often incomplete, and disease severity can vary considerably. Econs and McEnery described a large kindred, in which affected individuals presented with hypophosphatemia and/or rickets at variable ages,

ranging from early childhood to adolescence to adulthood; the mode of inheritance was autosomal dominant [145,146]. The phenotype at presentation may also vary, where children have short stature and rickets while adults have insufficiency fractures and skeletal pain, but no deformities of the lower extremities. Interestingly, some of the ADHR patients who manifested the phenotype in childhood had normalization of phosphate levels after puberty [145,147], and one patient presented with severe hypophosphatemia and osteomalacia after several uncomplicated pregnancies [148].

To determine the molecular cause of ADHR, Econs et al. used a positional cloning strategy and obtained linkage to a genetic locus on chromosome 12p13.3 in the previously described kindred [146], which subsequently resulted in different kindreds in the identification of heterozygous missense mutations in the *FGF23* gene (R176Q, R179Q, and R179W) [149]. The FGF23 mutations in ADHR patients replace one of two arginine (R) residues within the RXXR motif, a cleavage site for a subtilisin-like proprotein convertase, thus rendering FGF23 resistant to cleavage and increasing the amount of intact biologically active FGF23 in the circulation [48,147,150–152]. Studies where mutant forms of FGF23 carrying known ADHR mutations were expressed in vitro, Western blot analysis predominantly detected the intact form of FGF23, while expression of wild-type FGF23 demonstrated both the intact and cleaved FGF23 species [48]. Similarly, expression of the mutant FGF23 in mice resulted in increased serum levels of biologically active intact FGF23 [153].

The ADHR phenotype is particularly pronounced in individuals with iron deficiency, such as with onset of menses during puberty or during the postpartum period. Serum iron levels are inversely correlated with intact and C-terminal FGF23 levels in patients with ADHR. In unaffected controls, only the C-terminal FGF23 levels show this correlation with iron levels, while intact levels do not [38]. FGF23 expression also increased when mice with ADHR were placed on iron-deficient diet. These animals exhibited reduced renal *Npt2a* and *Cyp27b1* expression with elevations in *Cyp24a1* [37]. Iron supplementation of iron-deficient adults with ADHR normalized serum FGF23 levels and increased serum phosphate levels [154]. These studies demonstrate a role for iron in the maintenance of phosphate homeostasis. They also highlight the importance of the ratio of intact to C-terminal FGF23 in the regulation of phosphate balance.

### 3.2.3 Autosomal recessive hypophosphatemic rickets

The phenotype of autosomal recessive hypophosphatemic rickets (ARHR) resembles that of ADHR and XLH, where patients also show osteomalacia and rachitic skeletal changes early in life, leading to short stature. Like



patients with XLH, affected individuals may also develop dental defects and enthesopathies. The biochemical profile of ARHR is similar to that of ADHR and XLH, with hypophosphatemia, normal or low circulating levels of  $1,25(\text{OH})_2\text{D}$ , increased urinary phosphate excretion, and elevated serum levels of alkaline phosphatase and FGF23 (Table 64.2).

ARHR can be caused by homozygous or compound heterozygous inactivating genetic mutations in the *DMP1* (ARHR type 1), *ENPP1* (ARHR type 2), or *FAM20C* (ARHR type 3) genes. Lorenz-Depiereux et al. mapped the genetic lesion in two families with ARHR to chromosome 4q21, which comprises the *DMP1* gene [155]. Mutations have subsequently been identified that affect either the start codon, alter several other amino acid residues, or introduce splice site mutations [51,155]. Consistent with the human phenotype, the *DMP1*-null mouse exhibit a phenotype consistent with ARHR, including poor skeletal mineralization and rickets combined with low serum phosphate, elevated serum FGF23, and renal phosphate wasting [51]. The intact *DMP1* protein undergoes cleavage into 37- and 57-kDa fragments, where the 57-kDa fragment has been shown to be sufficient to rescue the *DMP1*-null mice from hypophosphatemia and to suppress FGF23 [156].

Loss-of-function mutations of the ecto-nucleotide pyrophosphatase/pyrophosphodiesterase 1 (*ENPP1*) gene have been implicated in ARHR [157]. *ENPP1* regulates skeletal mineralization by hydrolyzing ATP into pyrophosphate ( $\text{PPi}$ ), an inhibitor of mineralization [158]. The *Enpp1*-null mouse is characterized by hypophosphatemia, undetectable pyrophosphate levels, elevated FGF23 serum levels, as well as ectopic calcifications of the aorta, kidney, and spine [159,160]. The identification of *ENPP1* mutations in hypophosphatemic rickets demonstrates that this molecule regulates phosphate homeostasis and FGF23, in addition to its role in modulating skeletal mineralization. Patients with *ENPP1* mutations may present in infancy with generalized arterial calcification of infancy, with variable calcification of medium- and large-vessel arteries that can be life-threatening. These patients can also manifest with early-onset hearing loss, mineralizing enthesopathies, and ossification of the posterior longitudinal ligament of the spine [54,157,161]. During adolescence, serum FGF23 levels begin to rise, leading to hypophosphatemia and rickets; the severity of the rachitic phenotype is variable and correlates with serum FGF23 levels [162,163]. Although limited case reports show that conventional therapy with phosphate and calcitriol supplementation do not worsen arterial calcifications [162], the use of the anti-FGF23 antibody burosumab in an affected patient resulted in increased cardiac and vascular calcifications [164]. More recently, *Enpp1*-null mice treated with

humanized *ENPP1*-Fc prevented organ and cardiovascular calcifications, increased pyrophosphate levels, and improved growth and skeletal microarchitecture, pointing to a potential beneficial role for *ENPP1* enzyme replacement therapy in treating affected patients [165].

Compound heterozygous mutations of *FAM20C*, or the family with sequence similarity 20 member C, have been reported in siblings with hypophosphatemic rickets [166]. *FAM20C* is a kinase located in the endoplasmic reticulum and Golgi network and phosphorylates proteins with the SxE motif, which includes proteins like *DMP1* [167,168] (Fig. 64.3). Therefore, deletion of *FAM20C* may decrease the phosphorylation of *DMP1*, thus inhibiting *DMP1* expression or action and creating a *DMP1*-null phenotype [158]. Studies have also demonstrated that *FAM20C* acts to phosphorylate FGF23 at Ser180, which prevents glycosylation by *GALNT3* and thus renders FGF23 susceptible to cleavage by SPC proteases like furin [47]. Mutations in *FAM20C* increase the ratio of intact to C-terminal FGF23, therefore promoting urinary phosphate excretion. Patients with *FAM20C* mutations can have osteosclerosis, ectopic calcifications in the brain, and significant tooth decay. Interestingly, the phosphate wasting improves over time, and serum levels of phosphate are in the low normal range by the end of puberty [166]. The *Fam20c*-null mouse likewise develops hypophosphatemic rickets, with growth retardation and growth plate expansion [53]. Unlike the human phenotype, the *Fam20c*-null mouse does not have osteosclerosis. Ablation of *FAM20C* in osteogenic cell lines resulted in increased expression of FGF23 and decreased expression of *DMP1* [53].

### 3.2.4 Fibrous dysplasia

In fibrous dysplasia (FD), normal bone is replaced by fibrous tissue. It can affect one bone (monoostotic) or multiple bones (polyostotic) in the craniofacial, appendicular, or axial skeleton [169]. Children with this disorder usually present by 10 years of age with bone pain, fracture, or progressive deformity. FD can manifest on its own or be part of McCune-Albright syndrome, which is caused by somatic postzygotic mutations in *GNAS*, the gene encoding  $G_s\alpha$ , which mediates the actions of numerous G protein-coupled receptors that use the cAMP/PKA signaling pathway; [170] these missense mutations responsible for McCune-Albright syndrome affect only one of two amino acid residues, Arg201 or Gln227 [171]. Affected patients typically have café-au-lait spots and endocrinopathies reflecting abnormal signaling by GPCRs, which can include gonadotropin-independent precocious puberty, thyroid lesions with or without hyperthyroidism, growth hormone excess, hypercortisolism, or testicular lesions with Leydig and/or Sertoli cell hyperplasia [172].



FD patients can have elevated serum FGF23 levels and corresponding low or inappropriately normal serum 1,25(OH)<sub>2</sub>D levels [173]. The degree of urine phosphate wasting and disease burden correlates with FGF23 serum levels [174]. Although fibrous lesions in FD express FGF23, overt hypophosphatemia and classic hypophosphatemic rickets are not frequently observed. It has been shown that there is altered FGF23 cleavage due to decreased glycosylation and increased furin activity in bone marrow stromal cells isolated from FD subjects, thus possibly explaining the lack of a rachitic phenotype in FD [175]. These studies suggest that G<sub>s</sub>α plays a role in the regulation of FGF23 metabolism.

The bone pain that accompanies FD, often more severe in adults than children, can be treated with bisphosphonates. Treatment with bisphosphonates has also been shown to decrease serum FGF23 levels, where the decline in serum FGF23 correlates with an increase in serum 1,25(OH)<sub>2</sub>D levels [173]. However, there may be an increased risk of osteonecrosis of the jaw in FD patients on bisphosphonate therapy [176]. There is limited data on the efficacy of denosumab (anti-RANKL antibody) in therapy for FD. Administration of denosumab to fibrous dysplasia patients can decrease bone turnover markers, bone pain, and expansion of bony lesions. Thus, its use should be closely monitored in patients, as treatment with denosumab has been reported to cause worsening hypophosphatemia and secondary hyperparathyroidism [177]. Discontinuation of denosumab treatment can be complicated by rebound osteoclast activation, leading to hypercalcemia [178]. However, a pediatric patient with McCune-Albright syndrome/FD with frank hypophosphatemia and rickets, who was treated with burosumab, revealed improvement in mineral ion homeostasis, decreased fractures, and improved bone pain and strength [179].

### 3.2.5 Linear nevus sebaceous syndrome/epidermal nevus syndrome

Linear nevus sebaceous syndrome (LNSS), also known as epidermal nevus syndrome (ENS), is a rare, sporadic, and highly variable disorder that is characterized by papillomatous epidermal hyperplasia as well as abnormalities in multiple organ systems, including neurologic, ophthalmic, cardiovascular, and urogenital deformities [180]. LNSS/ENS is also associated with hypophosphatemic rickets due to elevated serum FGF23 levels, although the source of the FGF23 had not yet been elucidated [181,182]. Affected individuals can present with overtly low serum levels of 1,25(OH)<sub>2</sub>D [181]. In recent studies, whole-exome sequencing identified somatic postzygotic activating mutations of the *HRAS* or *NRAS* genes in affected skin and bone tissue from ENS patients with elevated serum FGF23 levels, suggesting that RAS signaling may be

important for the modulation of FGF23 [182]. Since the skin lesions have not been found to express FGF23 mRNA or protein, it is postulated that dysplastic bone lesions could be the source of the excess circulating FGF23.

## 3.3 Genetic hypophosphatemic disorders with low FGF23 levels

### 3.3.1 NPT2a mutations

The type II sodium phosphate cotransporter (NPT2a, *SLC23A1*) is expressed exclusively in the brush border membrane of renal proximal tubular cells. Ablation of *Npt2a* in mice leads to increased renal phosphate excretion, resulting in hypophosphatemia and a compensatory decrease in serum FGF23, as well as an increase in serum 1,25(OH)<sub>2</sub>D levels, which increases intestinal calcium absorption and thus hypercalcemia, hypercalciuria, and leads to low PTH levels [183,184]. The age-dependent increase in serum 1,25(OH)<sub>2</sub>D levels corresponded with the resolution of rickets in these mice [183].

Two heterozygous mutations in the *SLC34A1* gene (A48P and V147M) were identified in patients, who presented with idiopathic hypophosphatemia, recurrent urolithiasis, and skeletal demineralization [185]. Co-expression studies in *Xenopus laevis* oocytes revealed that the mutant NPT2a resulted in a dominant negative effect of the mutant proteins [185]. Heterozygous mutations in the *SLC34A1* gene (91del7, A133V, H568Y) have also been reported in a cohort of patients with recurrent kidney stones [186]. Despite the nephrolithiasis, these patients did not manifest hypercalciuria, hypophosphatemia, or increased urinary phosphate excretion [186]. A cohort of children diagnosed with idiopathic infantile hypercalcemia was found to have autosomal recessive mutations in *SLC34A1* [88]. Most of these infants presented with severe hypercalcemia, profound renal phosphate wasting, and suppressed serum PTH. Approximately half of these patients had elevated serum 1,25(OH)<sub>2</sub>D levels during hypercalcemia. Metabolic abnormalities resolved with phosphate supplementation [88], which is thought to lower 1,25(OH)<sub>2</sub>D levels, to prevent absorptive hypercalciuria in affected patients, and to thereby prevent nephrocalcinosis. However, mice lacking *Npt2a* fed high-phosphate diets with or without high calcium were more likely to develop renal calcifications compared to wild-type mice fed a similar diet, suggesting optimization of dietary content of phosphate and calcium is needed in treating affected individuals [187].

### 3.3.2 NPT2c mutations

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH) is a rare autosomal recessive that was first

described in a large consanguineous Bedouin kindred in the mid-1980s [188,189]. Biochemical features in the affected family members are similar to those in mice with homozygous ablation of *Npt2a*, namely affected patients have low serum phosphate and increased urinary phosphate excretion, leading to decreased FGF23 levels, rickets/osteomalacia, and short stature. The appropriate elevation of serum 1,25(OH)<sub>2</sub>D levels in these patients increases intestinal calcium absorption and suppresses serum PTH levels, thus resulting in hypercalciuria [188,189] (Table 64.2). Linkage studies using this and other smaller families lead to the identification of the genetic locus of this disease, and the subsequent identification homozygous and compound heterozygous mutations in the gene encoding NPT2c (*SLC34A3*) [190,191]. Reported human mutations in *SLC34A3* characterized using in vitro expression systems demonstrated that these mutations compromised the activity, expression, or stability of NPT2c [192].

Furthermore, these and other HHRH kindreds with documented *SLC34A3* mutations exhibit a significant increase in the occurrence of kidney stones. Thus, 46% of subjects with *SLC34A3* mutations on both alleles manifested nephrocalcinosis compared with 6% of non-affected family members [193]. Even heterozygous carriers of *SLC34A3* mutations have a higher frequency of renal calcifications that is intermediate to that seen in subjects with homozygous/compound heterozygous mutations and subjects without such mutations. The likelihood of renal calcifications increased with the severity of hypophosphatemia and decreased tubular reabsorption of phosphate [193]. The identification of *SLC34A3* mutations in HHRH has documented that NPT2c plays an important role in the regulation of phosphate homeostasis [194].

### 3.4 Acquired hypophosphatemic disorders with elevated FGF23 levels

#### 3.4.1 Tumor-induced osteomalacia

Tumor-induced osteomalacia (TIO), also known as oncogenic osteomalacia, is a rare paraneoplastic disorder that manifests with often profound urinary phosphate excretion and thus hypophosphatemia, low or inappropriately normal circulating levels of 1,25(OH)<sub>2</sub>D [195] (Table 64.2). In most cases, the cause of these mineral ion and hormonal abnormalities has been attributed to tumors secreting FGF23 [35,196] and consequently elevated levels of FGF23 in the circulation [46,197]. In patients whose FGF23 levels are not elevated, increased circulating levels of other phosphatonins such as secreted frizzled related protein-4 (SFRP4), matrix extracellular phosphoglycoprotein (MEPE), or fibroblast growth factor 7 (FGF7), have

been reported to cause TIO [198–200]. However, while the infusion of SFRP4 into rats results in increased phosphaturia and hypophosphatemia without changes in serum levels of 1,25(OH)<sub>2</sub>D or renal expression of 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase [199], mice with ablation of *Sfrp4* revealed no abnormalities in mineral ion homeostasis [201]. More recently, phosphaturic tumors expressing fusion products FN1-FGF1 or FN1-FGFR1 have been identified, supporting an important role of FGFR1 signaling in phosphate homeostasis [39].

TIO patients have osteomalacia and increased incidence of fractures. Children may manifest with gait disturbances and growth retardation. Tumors found in patients with TIO are predominantly of mesenchymal in origin and can be very slow growing and difficult to localize, often presenting in obscure or inaccessible areas such as the nasopharynx or mandible. Functional imaging is important for detection and localization of tumors. Since somatostatin receptors are often highly expressed on phosphaturic tumors, imaging modalities using radioisotopes to target these receptors are effective in localizing tumors. The <sup>68</sup>Ga-DOTATE PET/CT has the highest sensitivity and specificity for detecting tumors [202,203]. Also, <sup>18</sup>FDG-PET/CT can aid in detection of lesions of high metabolic activity. The duration of symptoms prior to diagnosis can range from a few months to 19 years [204]. Cure for this disorder is surgical resection. However, if the tumor cannot be found or completely resected, therapy with calcitriol and phosphate supplementation can help improve the metabolic and bone abnormalities [204]. In cases of incurable TIO due to FGF23 secreting tumors, the humanized anti-FGF23 antibody burosumab has been used to increase serum phosphate levels and improve osteomalacia [205].

### 3.5 Genetic hyperphosphatemic disorders

#### 3.5.1 Tumoral calcinosis

Tumoral calcinosis is a rare disorder characterized by hyperphosphatemia, leading to deposition of calcium phosphate crystals in soft tissue, bone, and periarthral spaces. Cases of tumoral calcinosis in North America have largely been reported in African Americans. However, case reports have also been published identifying this disorder in patients from Turkey, Iran, Kenya, and Saudi Arabia [206–209]. Tumoral calcinosis is predominantly sporadic but can be autosomal recessive. In addition to elevated serum phosphate levels, biochemical consequences of tumoral calcinosis include increased urinary TmP/GFR and inappropriately normal or increased serum 1,25(OH)<sub>2</sub>D levels (Table 64.2). Serum calcium and

PTH levels are usually normal. The elevated calcium phosphate product results in the deposition of calcific tumors. Patients can present with a wide range of involvement, from no calcified lesions to large and painful masses [210]. There is a predilection for these calcified lesions to occur in the hips, elbows, shoulders, and scapulae [211]. There have also been reports of lesions in the vasculature and eye as well as dental abnormalities with short roots and pulp stones [211]. Patients can also present with hyperostosis or systemic inflammation, manifesting with fevers or polyarthritis, along with high serum C-reactive protein levels, thought to be due to an inflammatory response from macrophages trying to engulf calcific lesions [210].

Outcomes of treatment for tumoral calcinosis can be variable. It has been suggested that the fibrous encapsulation of the calcified tumors can make phosphate deprivation therapy ineffective for decreasing the size of the lesions [211]. Calcific masses may be surgically excised when appropriate, though regrowth of lesions may occur because they may be poorly circumscribed [211]. However, phosphate deprivation with a diet low in calcium and phosphorus combined with aluminum hydroxide containing antacids has resulted in biochemical, clinical, and radiographic improvement in case reports of patients with tumoral calcinosis [212,213]. Furthermore, administration of the non-calcium phosphate binder sevelamer in combination with acetazolamide can also reduce serum phosphate levels and decrease the size of calcified lesions [214,215]. It is recommended that phosphate binders should be given prior to each meal and that dietary phosphate be restricted to 600–800 mg/day. Preclinical studies demonstrated that treatment of mice or rats with chronic renal disease and hyperphosphatemia or mice with tumoral calcinosis (Galnt3- or Fgf23-null mice) with a novel NPT2a small molecule inhibitor decreases serum phosphate levels and increases phosphaturia, suggesting that this inhibitor has the potential to be used as a treatment for tumoral calcinosis [216,217].

The genetic defects underlying tumoral calcinosis include mutations in the genes encoding FGF23, GALNT3, or *Klotho*, all of which lead to a defect in FGF23 action, and thus hyperphosphatemia and elevated 1,25(OH)<sub>2</sub>D levels. The biochemical phenotype for this disorder is therefore a mirror image of hypophosphatemic rickets, where there is an excess of FGF23 action. Biallelic inactivating FGF23 mutations have been reported. Full-length FGF23 is either destabilized or not secreted due to retention in the Golgi apparatus [218,219]. In these cases, affected individuals had increased circulating levels of the biologically inactive C-terminal FGF23 fragments and decreased circulating levels of intact FGF23 [218,219].

UDP-N-acetyl- $\alpha$ -D-galactosamine:polypeptide N-acetylgalactosaminyl-transferase 3 (GALNT3) is responsible for the mucin-type O-glycosylation of FGF23 at a subtilisin-like pro-protein convertase recognition sequence motif, which is essential for the secretion of biologically active intact FGF23 [220]. Homozygous or compound heterozygous *GALNT3* mutations have been reported in patients with defects in the posttranslational modification of FGF23 resulting in tumoral calcinosis [221,222]. Circulating levels of intact FGF23 are normal or low, while C-terminal fragments of FGF23 are elevated [221,223]. Compound heterozygous *GALNT3* mutations have also been reported in patients who presented with hyperostosis of the tibia combined with hyperphosphatemia and elevated 1,25(OH)<sub>2</sub>D levels, suggesting hyperostosis–hyperphosphatemia syndrome may be a continuum of the tumoral calcinosis phenotype. Like other cases of tumoral calcinosis, this patient also had elevated circulating levels of C-terminal FGF23 fragments and low levels of intact FGF23 [224].

Homozygous mutations in the *Klotho* gene have been reported in a patient who presented with severe vascular and soft tissue calcifications, as well as increased serum levels of calcium, PTH and 1,25(OH)<sub>2</sub>D [225]. The phenotype is consistent with that of the *Klotho*-null mouse, which is also characterized by ectopic soft tissue and vascular calcifications. Both mice and humans with homozygous *Klotho* mutations have elevated circulating FGF23 levels. Analysis of the His193Arg change demonstrated that this *Klotho* mutation caused destabilization of the *Klotho* protein, thereby decreasing its production and secretion [225]. Consequently, in the absence of *Klotho*, FGF23 was unable to bind to the FGFR1 demonstrating the importance of this co-receptor in FGF23-mediated phosphate and 1,25(OH)<sub>2</sub>D homeostasis.

## 3.6 Disorders of altered phosphate load

### 3.6.1 Phosphate deprivation

Although low serum phosphate due to poor dietary intake is rare, severe hypophosphatemia has been reported in children with kwashiorkor or marasmic kwashiorkor [226]. Up to 86% of children admitted for kwashiorkor in the Kenyatta National Hospital in Nairobi had hypophosphatemia. Following nutritional intervention, serum phosphate levels decreased further during the first 2 days of treatment [226], suggesting a refeeding syndrome. Serum phosphate levels of less than 1.0 mg/dL were described in a cohort of children presenting with kwashiorkor in Malawi, where the severity of hypophosphatemia correlated with the fatality rate [227].

In rats gavaged with a low phosphate diet, serum phosphate levels declined by 1 h post-gavage,

associated with increased renal tubular phosphate reabsorption. Thyroparathyroidectomized rats demonstrated similar rates of renal phosphate uptake during phosphate deprivation, suggesting that the renal adaptation to hypophosphatemia is independent of PTH [228]. Similarly, healthy male subjects placed on a low phosphate diet with aluminum hydroxide exhibited increased TmP/GFR, unchanged serum PTH levels, as well as compensatory increased serum 1,25(OH)<sub>2</sub>D levels [60]. In another study, administration of a low phosphate diet to healthy males and females suppressed urinary phosphate excretion and decreased serum PTH levels [16].

### 3.6.2 Gastrointestinal malabsorption

Hypophosphatemia may result from chronic ingestion of high doses of phosphate binder medications including calcium acetate or sevelamer [229]. Generally, mild or moderate doses of phosphate binders do not result in phosphate depletion. Use of medications such as aluminum or magnesium containing antacids has also been reported to cause low serum phosphate levels, leading to osteomalacia and myopathy [230,231]. The combination of hypophosphatemia with increased 1,25(OH)<sub>2</sub>D levels impaired skeletal mineralization. Healthy subjects who ingested a combination of low phosphate diet and antacids for 3 months had serum phosphate levels at as low as 1 mg/dL [230].

Disorders where there is a decreased vitamin D adsorptive capacity in the gut can also lead to lower serum phosphate levels. In small bowel resection and Crohn's disease, there is reduced surface area for vitamin D adsorption [232,233]. Also, vitamin D deficiency can result from steatorrhea in cystic fibrosis or chronic pancreatitis [234]. Vitamin D deficiency can lead to secondary hyperparathyroidism, where both processes contribute to the mild to moderate hypophosphatemia observed in malabsorptive disorders. In response to the elevation in PTH levels, serum levels of 1,25(OH)<sub>2</sub>D can also increase. Patients who underwent bariatric surgery have higher serum 1,25(OH)<sub>2</sub>D levels by 3–6 months post-procedure, where levels are significantly increased by 12 months. Circulating 1,25(OH)<sub>2</sub>D levels are higher in patients receiving Roux-en-Y gastric bypass or biliopancreatic diversion with duodenal switch as compared with gastric banding [235].

## 4. Conclusion

Characterization of molecular basis of genetic and acquired disorders of phosphate homeostasis has resulted in a greater understanding of the molecular regulation of phosphate balance. Phosphate is modulated by an

interplay of mineral hormones, where the manifestations of disruptions of these hormones have allowed for better insights into the molecular and skeletal processes that these hormones regulate. While significant advances have been made in deciphering the genetic and molecular basis for disorders of phosphate homeostasis, major gaps still exist in the availability of appropriate treatments of the complications of these disorders.

## 5. Summary points

- The proximal tubule of the kidney is the major site of phosphate reabsorption.
- Phosphate homeostasis is regulated by FGF23, 1,25(OH)<sub>2</sub>D, and PTH.
- Phosphate disorders with high serum FGF23 levels are characterized by hypophosphatemia and inappropriately normal serum 1,25(OH)<sub>2</sub>D levels, leading to rickets and osteomalacia. They include genetically inherited diseases such as XLH and acquired disorders such as TIO.
- Phosphate disorders that have low serum FGF23 levels are caused by mutations in renal sodium phosphate transporters that lead to increased phosphaturia and thus hypophosphatemia.
- Hyperphosphatemic disorders such as tumoral calcinosis result in formation of debilitating ectopic calcium phosphate deposits in soft tissue.
- Poor dietary intake of phosphate or gastrointestinal malabsorption of phosphate can lead to hypophosphatemia.

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# The hypocalcemic disorders

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## OBJECTIVES

- Review calcium physiology and regulation by vitamin D and parathyroid hormone.
- Describe the clinical manifestation of hypocalcemia.
- Present the differential diagnosis of hypocalcemia: abnormalities in parathyroid hormone availability, resistance to actions of parathyroid hormone and parathyroid hormone-independent hypocalcemia.
- Discuss treatment of hypocalcemia.

## 1. Introduction

Calcium is maintained within a narrow range by calcitropic hormones, including parathyroid hormone (PTH), and vitamin D. Hypocalcemic disorders are classified by their functional etiologies and discussed in relationship to primary homeostatic disturbances including inadequate PTH secretion, resistance to PTH action, or PTH independence. Identifying the underlying etiology of hypocalcemia is important, as therapeutic options and treatment goals vary depending on the specific cause.

## 2. Physiology of hypocalcemia

Hypocalcemia refers to an abnormally low concentration of circulating ionized calcium. Regulatory mechanisms maintain the concentration of ionized calcium

within a remarkably narrow range of 4.48–5.28 mg/dL (1.12–1.32 mmol/L) in whole blood [1]. The ionized fraction of total serum calcium is estimated to be ~ 50%, with the remainder of the total serum calcium bound to serum proteins, most notably albumin, and to a lesser extent complexed with anions, such as citrate or sulfate. Only the ionized fraction of total serum calcium is physiologically important, and it is this component that is regulated on a minute-to-minute basis.

Although it is possible to measure ionized calcium in large clinical laboratories, the specimen must be obtained anaerobically and analyzed promptly. Therefore, total serum calcium is often used as an indirect assessment of the ionized calcium fraction. A decrease in serum protein concentrations (particularly albumin) often results in reduced total serum calcium concentrations but does not affect the ionized calcium concentration. Such patients are asymptomatic, displaying none of the signs or symptoms of hypocalcemia. These findings are often present in patients with nephrotic syndrome, chronic illness, malnutrition, cirrhosis, and volume overexpansion. Occasionally, ionized calcium levels may transiently fall when protein losses are substantial (e.g., in severe nephrotic syndrome and protein-losing enteropathies), presumably due to massive losses of both ionized and protein-bound calcium. Several clinical guidelines have been suggested, which correct the effect of a reduced serum albumin on total serum calcium concentration. One commonly cited rule of thumb is to add 0.8 mg/dL to the total serum calcium for every 1 g/dL decline in serum albumin below 4.0 g/dL. However, these estimates are somewhat inaccurate under many circumstances, and it may be preferable to directly determine the ionized calcium concentration in the setting where the total serum calcium measure is thought to not accurately



reflect the concentration of ionized calcium [2]. Additionally, acidosis decreases the binding of calcium to protein, and alkalosis increases protein binding. As such, alkalosis leads to a decrease in ionized calcium concentration as the protein-bound fraction increases [3], which may precipitate hypocalcemic symptoms.

## 2.1 Parathyroid hormone in the acute defense of serum calcium concentration

PTH is secreted by the parathyroid glands in response to a fall in ionized serum calcium concentration [1]. The relationship between decrements in ionized calcium within the physiological range and increments in PTH secretion is quite steep, permitting rapid and substantial changes in PTH secretion in response to minor fluctuations in ionized calcium [1]. This response is mediated by the seven-transmembrane-domain, G protein-coupled calcium-sensing receptor (CaSR), which is expressed in the parathyroid glands and in a variety of other tissues. A rise in ionized calcium suppresses PTH secretion by activating this receptor [1].

PTH acts to regulate ionized calcium through its effects in three principal target tissues: the bone, kidney, and, indirectly, the intestine. The cellular actions of PTH are mediated by the PTH receptor, also a seven-transmembrane-domain, G protein-coupled receptor [4]. Downstream signaling from the PTH receptor involves activation of both protein kinase A (PKA)-dependent and protein kinase C (PKC)-dependent pathways [5,6]. In bone, PTH indirectly increases bone resorption, by stimulating osteoblastic and osteocytic expression of the receptor activator of nuclear factor kappa-B (RANK) ligand and inhibiting expression of the decoy receptor osteoprotegerin (OPG), thus increasing the RANK ligand/OPG ratio. When RANK ligand binds to its receptor RANK, expressed on the surface of osteoclast progenitors, osteoclastogenesis is promoted, leading to increased bone resorption and liberation of calcium from the mineralized matrix into the circulation [7–9].

The renal effects of PTH to defend serum calcium occur within minutes. PTH increases calcium reabsorption in the distal tubule. This effect is greatest in the distal convoluted tubule, where a sodium/calcium exchanger is regulated by PTH [10]. The transient receptor potential vanilloid 5 (TRPV5) calcium channel, another mediator of calcium reabsorption in the distal tubule, is also regulated by PTH. PTH increases the calcium inward flux of TRPV5, a PKA-mediated function [11], decreases endocytosis of TRPV5, a PKC-mediated function [12], and increases expression of TRPV5 [13]. Channel function of TRPV5 is also enhanced by the protein KLOTHO, which serves to preserve its integration in the membrane due to exposure of glycosaminoglycan-binding sites [14]. In the

proximal renal tubule, PTH acts via a cyclic adenosine monophosphate (cAMP)-dependent mechanism to decrease phosphate reabsorption, resulting in phosphaturia. PTH effects this change by prompting the removal of sodium/phosphate cotransporters from the renal tubular apical membrane [15]. These two effects both serve to acutely increase serum calcium; the combination of reduced renal calcium excretion combined with decreased serum phosphate levels favors an increase in ionized calcium.

The third site of action of PTH in the defense of serum calcium is the intestine. This is an indirect effect, described in detail in the following, and is a consequence of the ability of PTH to stimulate the renal production of 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ).

## 2.2 Vitamin D in the long-term maintenance of eucalcemia

Long-term eucalcemia is maintained, in large part, via the vitamin D endocrine system. This system operates in the classic manner of a steroid hormone, resulting in *de novo* protein synthesis directed by vitamin-D-responsive genes as discussed in detail in other sections. As noted before, acute changes in serum ionized calcium levels are sensed by G protein-coupled CaSRs located within the parathyroid cell membrane. PTH acts rapidly to correct a fall in serum calcium, and a sustained increase in PTH also stimulates production of  $1,25(\text{OH})_2\text{D}$ , which enhances intestinal calcium absorption. PTH mediates this change by promoting the increased expression of *CYP27B1*, which encodes the catalytic component of the renal 25-hydroxyvitamin D ( $25(\text{OH})\text{D}$ ) 1- $\alpha$ -hydroxylase ( $1\alpha\text{-OHase}$ ) enzyme complex, located in the inner mitochondrial membrane of renal tubular cells (see Chapter 8). A number of physiological studies have demonstrated increased production of  $1,25(\text{OH})_2\text{D}$  in animals that were administered PTH [16,17] and decreased production following parathyroidectomy [18]. PTH also acutely regulates the  $1\alpha\text{-OHase}$  enzyme complex by altering the phosphorylation state of the associated ferredoxin molecule, which serves to donate electrons to the catalytic complex [19]. A low extracellular calcium concentration can directly stimulate  $1\alpha\text{-OHase}$  activity in the absence of PTH as described in para-thyroidectomized rats [20] and in hypo-parathyroid humans [21].

Dietary calcium is absorbed mainly in the small intestine. When the intraluminal concentration of calcium is high, calcium absorption occurs primarily via a passive non-saturable paracellular pathway [22]. In states of decreased circulating calcium with a compensatory rise in PTH, increased synthesis of  $1,25(\text{OH})_2\text{D}$  results in greater circulating levels of the metabolite, which gain access to specific vitamin D receptors (VDRs). The

hormone–receptor complex then binds to vitamin D response elements in the regulatory regions of target genes to promote active absorption of calcium via transcellular pathways (see Chapters 10–13). Long-term control of calcium homeostasis has been thought to be classically mediated by  $1,25(\text{OH})_2\text{D}$  induction of the calcium channel TRPV6 [23] and the intestinal 9 kDa calcium-binding protein, calbindin-D9k, which is thought to play a role in vitamin D–mediated increases in calcium absorption in the jejunum and duodenum [24,25]. These proteins, however, may not be necessary for calcium absorption because mice in which both these proteins have been deleted still demonstrate substantial active intestinal calcium transport [26,27]. Rapid, non-genomic actions of  $1,25(\text{OH})_2\text{D}$ -mediating calcium transport across intestinal mucosa have also been described [28].

During vitamin D deprivation, the initial decline in ionized calcium results in secondary hyperparathyroidism, which maximizes  $1,25(\text{OH})_2\text{D}$  production and initially allows for maintenance of eucalcemia. Eventually, this compensatory mechanism fails, and intestinal calcium absorption is sufficiently compromised such that frank hypocalcemia develops. This may be compounded by an induced resistance to PTH seen in severe vitamin D–deficient states [29,30].

In children with hereditary resistance to vitamin D due to loss-of-function variants in the vitamin D receptor, the compensatory changes described before are disrupted because of nonfunctional VDRs that result in the inability of  $1,25(\text{OH})_2\text{D}$  to signal to the nucleus [31]. Untreated patients with VDR variants can have severe hypocalcemia, leading to convulsions, coma, and death, as this homeostatic system is effectively absent (see Chapter 68).

The vitamin D system has further complexities that are currently not well understood. For example, some children with vitamin D deficiency (defined by low  $25(\text{OH})\text{D}$  levels) manifest hypocalcemia even when circulating  $1,25(\text{OH})_2\text{D}$  levels are elevated. Possible explanations for this phenomenon include decrements in expression of calbindin during hypocalcemia or a requirement for other circulating vitamin D metabolites, which have yet to be identified. Whether changes in the concentration of VDR levels play a role in this “resistant” state is not clear, as receptor levels have been reported to increase, decrease, or not change with manipulation of the ambient calcium concentration [32–34] (see Chapters 62–64).

### 2.3 Biochemical changes induced by hypocalcemia

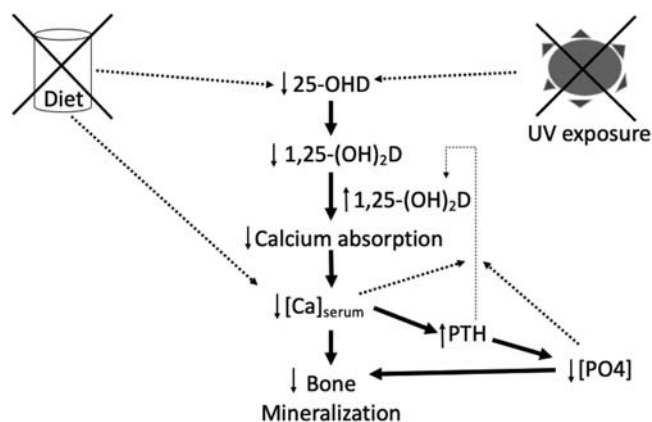
As noted before, the most rapid response to hypocalcemia is secretion of PTH. In addition to increasing

serum calcium levels, PTH stimulates renal phosphate (Pi) excretion. The fall in serum phosphate may, however, be compensated by sufficient mobilization of phosphate (as well as calcium) from bone, so that circulating phosphate remains largely unchanged. The principle of mass action is thought to maintain the stability of the  $\text{Ca} \times \text{Pi}$  ion product in the blood. Therefore, local concentrations of the two major mineral components of hydroxyapatite (Ca and Pi) can influence the rate of movement in and out of the mineral phase of bone.

Thus, a fall in ionized Ca would favor an increase in serum phosphate concentration. In aggregate, however, sustained hypocalcemia usually results in a biochemical picture of secondary hyperparathyroidism, elevated  $1,25(\text{OH})_2\text{D}$  levels, and variable changes in serum phosphate. If hypocalcemia develops in the setting of diminished or absent PTH function, serum phosphate is usually elevated, owing to decreased renal phosphate excretion. In this instance, treatment with  $1,25(\text{OH})_2\text{D}$  would also increase serum phosphate because this metabolite enhances intestinal phosphate absorption. The effect of hypocalcemia on circulating vitamin D metabolites is complex. An increase in the biosynthesis of  $1,25(\text{OH})_2\text{D}$  occurs, as reviewed before. This is largely secondary to the induced increase in circulating PTH but can be a direct consequence of the fall in calcium ion concentration. It has been determined that calcium deprivation results in a general increase in turnover of the parent vitamin D metabolite,  $25(\text{OH})\text{D}$ , such that vitamin D stores are depleted at a more rapid rate than normal [35]. The clinical implication of this finding is that susceptibility to vitamin D deficiency may be greater in the setting of concomitant calcium deprivation (see Fig.65.1).

### 3. Clinical manifestations of hypocalcemia

Manifestations of hypocalcemia are related to increased neuromuscular irritability [36]. Tetany is the classic sign of hypocalcemia, yet it is variable in presentation. Paresthesia often occurs first around the mouth or in the fingertips and may progress to overt spasm of the muscles of the face and extremities, the latter typified by carpopedal spasm. More subtle presentations have included complaints of writer’s cramp or generalized stiffness. Children with tetanic laryngospasm due to hypocalcemia have been mistakenly diagnosed with croup [37]. Infants are more likely than adults to present with jitteriness or twitching, which can progress to overt tonic–clonic seizures. Lethargy and cyanosis have also been described in this age group. The term latent tetany refers to signs elicitable with provocative stimuli such as ischemia (Trousseau test) or percussion (e.g., of the facial



**FIGURE 65.1** Mechanisms of the development of osteomalacia and rickets. Deficiency of vitamin D intake and/or limited ultraviolet light exposure lead to limited vitamin D stores as reflected by a decreased circulating 25(OH)D level. Reduced availability of this substrate is presumed to limit 1,25(OH)<sub>2</sub>D production, resulting in impaired intestinal calcium absorption. Calcium availability for skeletal mineralization is subsequently compromised, and secondary hyperparathyroidism, with concomitant hypophosphatemia, occurs. Restricted dietary calcium intake can also result in a similar pathophysiology. Increased turnover of vitamin D in the calcium-deficient state may result in a greater risk of vitamin D insufficiency. The paradox of elevated levels of 1,25(OH)<sub>2</sub>D in these disorders is well recognized. PTH, parathyroid hormone.

nerve to elicit Chvostek's sign). Neither the degree of hypocalcemia nor the rapidity with which it develops necessarily correlates with clinical manifestations. Many individuals with chronic, mild hypocalcemia may be entirely asymptomatic, as can be seen in congenital hypoparathyroidism.

Hypomagnesemia or hyperkalemia may present with findings like those caused by hypocalcemia, which can be exacerbated in the setting of hypocalcemia. Conversely, hypermagnesemia or hypokalemia can mask symptoms in a hypocalcemic individual [38]. Abnormalities of cardiac repolarization can occur with hypocalcemia resulting in a prolonged QT interval on the electrocardiogram (EKG). The QT interval corrected for heart rate (QTC, which equals QT/(RR interval)<sup>1/2</sup>) is normally less than  $0.40 \pm 0.04$  s. This abnormality is not always present during hypocalcemia, and it may also be seen in hypokalemia. Cardiac failure may rarely occur in the setting of hypocalcemia [39]. Papilledema has also been reported [40].

Chronic hypocalcemia caused by calcium deficiency, due to inadequate intake/intestinal absorption or excessive renal losses, during skeletal growth may result in rickets, osteomalacia, and osteoporosis. While dental abnormalities have also been reported in long-standing untreated hypoparathyroidism [41], osteoporosis is uncommon in hypoparathyroidism. To the contrary, adults with hypoparathyroidism typically display increased bone density, associated with a low bone turnover state

[42]. Despite marked hypocalcemia, rickets is not seen in hypoparathyroidism, due to the absence of hypophosphatemia. Basal ganglia calcifications and cataracts, associated with a decreased calcium:phosphate ratio, are typical findings in long-standing hypoparathyroidism as well [43–45]. Abnormalities in the integument including dry skin, coarse hair, and a form of psoriasis that responds to normalization of the serum calcium concentration [46] have all been described in states of long-standing hypocalcemia.

## 4. Differential diagnosis of hypocalcemia

### 4.1 Classification

A rapid increase in PTH serves as the major defense against acute hypocalcemia. Thus, hypocalcemic disorders can be divided into disorders of decreased PTH availability, impaired PTH signaling, or PTH-independent hypocalcemia. While many of the etiologies of hypocalcemia are directly related to abnormalities in PTH, vitamin D metabolism is always involved, and vitamin D is often the cornerstone of therapy.

### 4.2 Hypocalcemia due to abnormalities of parathyroid hormone availability

A variety of congenital or acquired disorders can lead to developmental failure of the parathyroid glands, failure of functional hormone production, or destruction of the parathyroid glands (Table 65.1). These are all associated with variable degrees of hypocalcemia, usually with attendant hyperphosphatemia and undetectable or inappropriately low levels of circulating PTH.

### 4.3 Disorders of parathyroid gland formation

The condition 22q11.2 deletion syndrome (22q11.2DS) is the most frequent chromosomal microdeletion syndrome, with an estimated frequency of about 1/4000 in children under a year of age. One of the cardinal features is dysgenesis of the thymus and the parathyroid glands. The syndrome has been assigned several names in the past, including DiGeorge syndrome, velocardiofacial syndrome, and conotruncal anomaly face syndrome. The syndrome has a broad phenotype ranging from mild intellectual disabilities, congenital heart disease, parathyroid abnormalities, significant neurocognitive impairments, and risk for psychiatric disorders [47]. Tetany and seizures are common features of the early course of infants with 22q11.2DS with hypocalcemia reported as a feature in up to 60% of cases. Abnormalities in T cell function with subsequent increased risk for infection are also a

**TABLE 65.1** Hypocalcemia due to abnormalities of parathyroid hormone.

	Inheritance	Locus	Gene	OMIM	Associated abnormalities
<i>Disorders of the parathyroid gland</i>					
Di George syndrome type 1 (DGS1)	AD	22q11.2	del ( <i>TBX1</i> )	188400	Thymic hypoplasia with immune deficiency, conotruncal defects, cleft palate, dysmorphic facies, short stature, developmental delay, psychiatric disorders
Di George syndrome type 2 (DGS2)	AD	10p13-p14	del ( <i>NEBL</i> )	601362	
CHARGE syndrome	AD	7q21.11 8q12.2	<i>SEMAE3</i> <i>CHD7</i>	214800	Choanal atresia and malformations of the heart, inner ear, and retina (coloboma), poor growth, genital hypoplasia
Hypoparathyroidism, deafness, renal dysplasia (HDR)	AD	10p14	<i>GATA3</i>	146255	Deafness, renal dysplasia
Hypoparathyroidism, retardation, dysmorphism syndrome (HRDS)	AR	1q42.3	<i>TCBE</i>	241410	Growth retardation, infantile-onset hypoparathyroidism, developmental delay, dysmorphic facial features Severe proportionate short stature, cortical thickening with medullary stenosis of the tubular bones, craniofacial abnormalities, eye abnormalities Gracile bones with thin diaphysis, premature closure of basal cranial sutures, and microphthalmia
Sanjad-Sakati syndrome	AR	1q42.3	<i>TCBE</i>	244460	
Kenny-Caffey syndrome 1 (KCS1)	AD	11q12.1	<i>FAM111A</i>	127000	
Kenny-Caffey syndrome 2 (KCS2)	AD	11q12.1	<i>FAM111A</i>	602361	
Gracile bone dysplasia (GCLEB)					
Mitochondrial disease	AR	mtDNA	mtDNA	530000	Ophthalmoplegia, pigmentary degeneration of the retina, and cardiomyopathy Bone marrow failure (altered hematopoietic precursors), diabetes, malabsorption Myopathy, encephalopathy, lactic acidosis, stroke-like episodes, seizures, cortical blindness, hemianopsia, episodic vomiting Hypoglycemia, cardiomyopathy, myopathy with hypotonia, episodic vomiting, liver disease, peripheral neuropathy Hypoglycemia, lethargy, vomiting, seizures Recurrent hypoglycemia, rapidly progressive myopathy, cardiomyopathy Multiple congenital malformations (microcephaly, abnormal genitalia and nostril), mental retardation, adrenal insufficiency
Kearns-Sayre syndrome (KSS)	AR	2p23.3	<i>HADHA</i> , <i>HADHB</i>	540000	
Pearson marrow-pancreas syndrome	AR	1p31.1	<i>ACADM</i>	557000	
	AR	2p23.3	<i>HADHA</i>	609016	
Mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes syndrome (MELAS)		11q13.4	<i>DHCR7</i>	201450	
				609016	
Mitochondrial functional protein deficiency syndrome (MTPD)				270400	
Medium-chain acyl-CoA dehydrogenase deficiency (ACADM)					
Long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD deficiency)					
Smith-Lemli-Opitz syndrome (SLOS)					
Familial isolated hypoparathyroidism type 2 (FIH2)	AR, AD	6p24.2	<i>GCM2</i>	618883	
Hypoparathyroidism X-linked recessive (HYPX)	XLR	Xq27.1	Del/ins ( <i>SOX3</i> )	307700	

Continued



TABLE 65.1 Hypocalcemia due to abnormalities of parathyroid hormone.—cont'd

	Inheritance	Locus	Gene	OMIM	Associated abnormalities
<i>Disorder of parathyroid gland secretion</i>					
Maternal hypercalcemia					Early neonatal hypocalcemia; transient
Hypomagnesemia	AR	9q21.13	TRPM6	602014	Multiple genetic and acquired causes; inhibits parathyroid hormone (PTH) secretion and PTH sensitivity Hypercalciuria, nephrocalcinosis Hypocalciuria Progressive loss of kidney function, amelogenesis imperfecta Mild to moderate psychomotor retardation Progressive renal failure, nephrocalcinosis, and severe visual impairment, amelogenesis imperfecta - Hypokalemic metabolic alkalosis, hypocalciuria, abdominal pain, chondrocalcinosis Spells of incoordination and imbalance, often associated with progressive ataxia Hypertension, hypercholesterolemia Seizures, delayed psychomotor development, limited speech Significantly impaired intellectual development
Hypomagnesemia syndromes:	AD	11q23.3	FXYP2	154020	
Hypomagnesemia 1, intestinal (HOMG1)	AR	3q28	CLDN16	248250	
	AR	4q25	EGF	611718	
Hypomagnesemia 2, renal (HOMG2)	AR	1p34.2	CLDN19	248190	
	AD	10q24.32	CNNM2	613882	
Hypomagnesemia 3, renal (HOMG3)	AR	16q13	SLC12A3	263800	
	AD	12p13.32	KCNA1	160120	
Hypomagnesemia 4, renal (HOMG4)	AD, AR	10q24.32	Mt DNA	500005	
	AD	1p13.1	CNNM2	616418	
Hypomagnesemia 5, renal (HOMG5)			ATP1A1	618314	
Hypomagnesemia 6, renal (HOMG6)					
Gitelman syndrome (GTMNS)					
Episodic ataxia type 1 (EA1)					
Hypomagnesemia, hypertension, and hypercholesterolemia syndrome					
Hypomagnesemia, seizures, and mental retardation 1 (HOMGSMR1)					
Hypomagnesemia, seizures, and mental retardation 2 (HOMGSMR2)					
Hypermagnesemia					Inhibition PTH secretion via activation of the CaSR
Familial isolated hypoparathyroidism type 1 (FIH)	AR or AD	11p15.3	PTH	146200	
Autosomal dominant hypocalcemia type 1 (ADH1)	AD	3q13.3–21	CASR	601198	Variable degree of severity, hypomagnesemia, hypercalciuria; some variants associated with Bartter syndrome type V Hypomagnesemia, hypercalciuria
Autosomal dominant hypocalcemia type 2 (ADH2)		19p13.3	GNA11	615361	
<i>Parathyroid gland damage/destruction</i>					
Complication of neck surgery					May be transient
Infiltrative disease/heavy metals					Hemochromatosis, Wilson disease, metastases
Autoimmune hypoparathyroidism		Polygenic			Maybe associated with other autoimmune diseases
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED)	AD, AR	21q22.3	AIRE	240300	Adrenal insufficiency, mucocutaneous candidiasis, malabsorption, vitiligo, alopecia, hepatitis, pernicious anemia, hypogonadism, asplenia

**TABLE 65.1** Hypocalcemia due to abnormalities of parathyroid hormone.—cont'd

	Inheritance	Locus	Gene	OMIM	Associated abnormalities
<i>Resistance to PTH</i>					
Hypomagnesemia					As noted above
Severe vitamin D deficiency Vitamin D–dependent rickets, type 3	AD	7q22.1	<i>CPY3A4</i>	619073	Corrects with vitamin D replacement, many consider this to be the cause of pseudohypoparathyroidism type II Early-onset rickets, reduced serum levels of vitamin D metabolites, and no responsiveness to all forms of vitamin D
Pseudohypoparathyroidism Ia (PHP1a)	AD	20q13.32 (maternal)	<i>GNAS</i>	103580	Albright's hereditary osteodystrophy (AHO), obesity, other hormone resistance
Pseudopseudohypoparathyroidism (PPHP)	AD	20q13.32 (paternal)	<i>GNAS</i>	612463	AHO without obesity or hormone resistance
Pseudohyparathyroidism Ib (PHP1b)	Sporadic or AD	20q13.32 (maternal)	<i>GNAS</i> <i>STX16</i>	603233	Due to imprinting defects upstream of <i>G<sub>s</sub>α</i> promoter, nondysmorphic, may have TSH resistance
Pseudohypoparathyroidism Ic (PHP1c)	AD	20q13.32 (maternal)	<i>GNAS</i>	612462	Identical phenotype to PHP1a, normal <i>G<sub>s</sub>α</i> activity in erythrocytes, receptor coupling possibly affected

major feature of this disorder [48]. Deletions, translocations, and rearrangements of the chromosome 22q11.2 region occur in this disorder, with *TBX1*, a member of the t-box family of transcription factors, identified as the gene responsible for its characteristic craniofacial, parathyroid, and cardiac anomalies [49,50]. It is believed that contiguous genes, as well as a variety of other genetic modifiers, are responsible for the broad spectrum of findings that exist in patients with 22q11.2 deletion syndrome. Deletions at a second locus on chromosome 10p have been identified as the cause for a phenotypically similar condition, termed DiGeorge syndrome II [51].

Several other genes have been identified as critical to parathyroid development. Loss-of-function variants have been identified as the genetic basis for some human hypoparathyroid syndromes [52]. These include loss-of-function variants in the parathyroid-specific transcription factor, glial cells missing B (*GCMB*), usually manifesting as familial, isolated hypoparathyroidism [53]. The combination of familial hypoparathyroidism, sensorineural deafness, and renal dysplasia (Barakat syndrome) is an autosomal dominant disorder due to pathogenic variants in the *GATA3* transcription factor [54,55]. An autosomal recessive condition of hypoparathyroidism, mental retardation, dwarfism, tubular deformities of the long bones, eye defects, and dysmorphic features, once thought to be two separate disorders (Sanjad-Sakati syndrome and Kenny-Caffey syndrome type 1) are now known to be a single disorder

mediated by loss-of-function variants in the tubulin chaperone E gene [56]. Recently, a dominant form of Kenny-Caffey syndrome type 2 has been described, due to pathogenic variants in *FAM111A* [57,58], the function of which is not fully understood. Mitochondrial gene defects can also result in hypoparathyroidism [59,60]. X-linked recessive hypoparathyroidism has been observed in patients with variants that affect expression of *SOX3*, a transcription factor implicated in parathyroid gland development [61]. Indeed, many cases of so-called “idiopathic hypoparathyroidism” are likely related to the previously described disorders.

#### 4.4 Disorders of parathyroid hormone production or secretion

PTH is secreted and synthesized by a classic secretory pathway. The initial translation product is a prepropeptide, which requires cleavage of the amino-terminal pre- and pro-sequences before secretion. A family with autosomal dominant inheritance of hypoparathyroidism has been reported in which a missense variant Cys → Arg variant (C18R) results in an abnormal signal sequence and diminished uptake of prepro-PTH into the endoplasmic reticulum [62]. Another family with recessively inherited hypoparathyroidism has been reported in which the preprosequence is deleted by a splicing variant [63]. Also, a family with a homozygous variant resulted in secretion of a bioinactive PTH species but

with normal to elevated intact PTH levels, suggestive of PTH resistance [64].

Parathyroid glands with normal anatomic structure may fail to secrete PTH appropriately if calcium sensing is altered. The gene for the CaSR (*CASR*) has been mapped to chromosome 3 [65,66]. Ionized calcium is a ligand for this receptor, and receptor occupancy suppresses PTH secretion. Numerous individuals and families with a variety of activating variants of the *CASR* have been reported; the associated condition is referred to as autosomal dominant hypocalcemia type 1 (ADH1) [67,68]. The renal expression of the mutant CaSR results in hyper-calciuria, despite the hypocalcemia, and an increased risk for nephrolithiasis has been reported in individuals harboring one of these variants. Progression to chronic kidney disease in this setting is a concern, particularly if urinary calcium excretion is not carefully monitored. An acquired autoimmune disorder, in which autoantibodies activate the CaSR, can mimic ADH1 [69,70]. Recently, activating variants in *GNA11*, the downstream G protein responsible for CaSR action, has been identified as a cause of ADH2 [71]. The phenotype is similar to ADH1, although variants in *GNA11* may also be associated with impaired growth and the earlier development of intracranial calcifications [72].

Severe hypomagnesemia is associated with suppressed parathyroid secretion as well as decreased end-organ sensitivity, resulting in hypocalcemia [73,74]. Conversely, as magnesium is also a ligand for the CaSR, hypermagnesemia inhibits PTH secretion via CaSR activation [1]. Transient suppression of PTH secretion causing hypocalcemia in neonates may also occur with maternal hypercalcemia [75].

#### 4.5 Parathyroid gland damage/destruction

The autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED), also known as autoimmune polyglandular syndrome type 1, is an autoimmune disorder characterized by early development of hypoparathyroidism in association with primary adrenal insufficiency and mucocutaneous candidiasis. The majority of affected individuals will manifest hypocalcemia by the age of 10 [76,77]. In addition to adrenal insufficiency, one-third of the patients will develop other endocrine disorders such as diabetes mellitus, pernicious anemia, or premature ovarian failure. This disorder is now known to be associated with pathogenic variants in a gene (*AIRE*) encoding an autoimmune regulatory protein containing a zinc finger motif and is a candidate transcription factor [78,79,204]. Affected individuals frequently develop numerous autoantibodies including antibodies to NACHT leucine-rich-repeat protein 5, a tissue-specific

autoantigen involved in hypoparathyroidism and primary ovarian insufficiency [80,81] as well as activating autoantibodies to the CaSR [82]. Polygenic forms of autoimmune hypoparathyroidism also occur, presenting more frequently in adults than in children, and may be associated with anti-parathyroid and CaSR autoantibodies as well [69,70,83]. Depending on the action of the specific autoantibody, PTH secretion may be impaired without true destruction of the gland.

Given the close anatomic relationship of the parathyroid glands to the thyroid, complete or near-complete extirpation of the thyroid gland as part of the management of either Graves' disease or thyroid cancer can be complicated by destruction or vascular compromise of parathyroid tissue and varying degrees of hypoparathyroidism. With increasing specialization of surgery practices, this should be a rare complication of thyroid surgery and, with experienced thyroid surgeons, occurs with a frequency of less than 10%. However, surgical complication remains the most common etiology of hypoparathyroidism in adults [45]. Even when destruction of the parathyroid glands does not occur following neck surgery, so-called "stunned" parathyroids with transient declines of  $\sim 1$  mg/dL in total serum calcium are often observed in the first 24–48 h postoperatively. This is presumably due to transient vascular or physical damage to the glands. Some surgeons treat with a brief course of calcitriol or calcium or both to protect against acute hypocalcemia immediately following surgery. Considerable variability in the degree of hypoparathyroidism following neck surgery occurs, ranging from asymptomatic reduction in parathyroid reserve to frank tetany, requiring chronic therapy with vitamin D and calcium. Although the parathyroid glands are quite resistant to radiation, transient hypoparathyroidism following radioactive iodine treatment for hyperthyroidism has been described [84]. Although uncommon, malignant metastasis to the parathyroid glands resulting in hypoparathyroidism has been reported, usually in the setting of breast cancer [85]. It has been postulated that granulomatous involvement of the parathyroids in sarcoidosis can lead to hypoparathyroidism [86]. Patients with transfusion-dependent thalassemia can develop hypoparathyroidism due to hemochromatosis secondary to deposition of iron in the glands [87]. In Wilson's disease, hypoparathyroidism can occur, presumably because of copper deposition [88]. Finally, impaired parathyroid reserve has been reported in diabetic patients with uremia [89].

#### 4.6 Hypocalcemia due to resistance to the actions of parathyroid hormone

Peripheral tissue insensitivity or resistance to PTH is classically termed pseudohypoparathyroidism (PHP)

[90]. The characteristic biochemical manifestations of PHP are hypocalcemia and hyperphosphatemia, as in classic hypoparathyroidism; however, circulating levels of PTH are elevated, rather than low or undetectable. The renal tubule is the primary site of PTH resistance, although skeletal resistance may occur to variable degrees and in some cases varies with treatment status [91]. However, if the skeletal response to PTH is normal, lesions characteristic of hyperparathyroidism, including osteitis fibrosa cystica, can develop [92,93].

Normally, PTH stimulates renal cAMP production, and levels of cAMP increase in the urine following administration of the hormone. A direct correlation has been demonstrated between the degree of PTH resistance (as assessed by the failure of urine cAMP excretion or phosphate to change) and the ambient circulating PTH level [94]. This renal cAMP response provides the basis for a diagnostic test that allows partial classification of this heterogeneous group of disorders. Individuals with PHP that demonstrates a blunted urinary cAMP response have PHPI. Those that generate a normal cAMP response have PHPII.

PHPI has been further characterized into types Ia, Ib, and Ic. Type Ia describes those individuals with the Albright's hereditary osteodystrophy (AHO) phenotype, which includes short stature and large frame, broad faces, and shortened fourth metacarpals, and soft tissue calcifications. These individuals often have a pathogenic variant in *GNAS*, the  $\alpha$  subunit of the stimulatory guanine nucleotide-binding regulatory protein,  $Gs\alpha$  [95]. This regulatory protein couples membrane receptors to adenylate cyclase, thereby regulating receptor-dependent cAMP production. The presence of  $Gs\alpha$  in various cell types accounts for the generalized hormone resistance that may occur. For example, affected patients often have elevated thyrotropin (TSH) levels. Variable degrees of gonadotropin, antidiuretic hormone, adrenocorticotropin, growth hormone releasing hormone, and glucagon resistance have been described [96]. Patients also have early-onset obesity and reduced insulin sensitivity [97,98]. *GNAS* and adjacent genes are imprinted, resulting in expression of only the maternal allele in certain tissues (e.g., proximal renal tubule) and biallelic expression in other (e.g., bone, distal renal tubules). Thus, the inactivating variant must be evident in the maternal allele for the expression of the classic PHPIa phenotype, where PTH-mediated effects at the proximal tubule are blunted while renal calcium reabsorption at the distal tubule is undisturbed [99]. Individuals who inherit the mutant *GNAS* from their father do not have hormone resistance but do demonstrate the AHO phenotype, referred to as pseudopseudohypoparathyroidism (PPHP), suggesting that biallelic expression is necessary in certain skeletal tissues. Of note, obesity and insulin resistance are not seen in PPHP [97,98], thereby

associating those aspects of the PHPIa phenotype with expression of the maternal allele.

PHPIb refers to individuals with PTH resistance without the AHO phenotype. These patients lack generalized resistance to most other hormones, although TSH resistance may be present [100] and a case with short metatarsals has been reported [101]. Unlike PHPIa, PHPIb is not due to inactivating variants in *GNAS*. The *GNAS* locus contains three transcripts that encode expressed proteins and two noncoding transcripts that are regulated by a complex interaction of alternative splicing and imprinting [102]. In familial cases of PHPIb, variants on the maternal allele upstream of *GNAS* in control element genes such as *STX16* or *NESP55* result in abnormal imprinting and reduced  $Gs\alpha$  activity in the proximal renal tubules [99]. Sporadic PHPIb may also be due to loss of imprinting in differentially methylated regions within the *GNAS* complex or paternal uniparental disomy of 20q. Other genetic and epigenetic changes may alter the function of the protein in different tissues [103].

Patients phenotypically and biochemically identical to PHPIa, who demonstrate normal  $Gs\alpha$  protein activity in vitro, have been classified as having PHPIc. While the genetic cause in the majority of cases currently remains unknown, studies in patients with PHPIc have identified variants in exon 13 of *GNAS*, resulting in abnormal receptor coupling [104] or other epigenetic alterations [105]. Thus, it appears that variants in *GNAS* or in neighboring regions that may affect its imprinting can cause a spectrum of defects seen in type I PHP, and an overlap between the subcategories of Ia, Ib, and Ic is likely to exist. Additionally, pathogenic variants in *PRKARIA*, which encodes the regulatory subunit of protein kinase A, and *PDE4D*, which encodes phosphodiesterase type 4, cause acrodysostosis, a rare skeletal disorder that may also exhibit PTH resistance and has features overlapping with PHPIa [106–108]. Therefore, a new classification system has been recently proposed under the umbrella of inactivating PTH/PTHrP signaling disorders [109].

In contrast to type I PHP, type II PHP is characterized by isolated resistance to the phosphaturic effects of PTH, while cAMP generation in response to PTH administration is normal. It has been suggested that this is due to variable defects distal to cAMP generation in the cascade of hormone action. AHO is absent, and no distinct skeletal phenotype is evident; various autoimmune findings have been described in some patients. This entity is quite rare, and some have questioned whether it exists as a distinct entity. It has been postulated that PHP II is a variant of severe vitamin D deficiency with associated PTH resistance because there have been numerous cases of reporting return of PTH sensitivity following vitamin D and calcium replacement [110,111].



## 4.7 Hypomagnesemia

Magnesium is necessary for release of stored PTH, and thus, magnesium deficiency can interfere with parathyroid secretion and function [73,74]. Serum magnesium levels are usually moderately to severely depressed (below the range of 1.0–1.4 mg/dL) before this occurs. Despite hypocalcemia, PTH levels may be inappropriately low or only modestly elevated, and tetany refractory to calcium supplementation can ensue. Hypomagnesemia, per se, may cause tetanic symptoms, but concomitant hypocalcemia is frequently present. Insufficient PTH secretion is the most widely accepted cause of refractory hypocalcemia in magnesium deficiency [73], although resistance to the calcemic actions of PTH and vitamin D may also play a role [112]. Impairment of vitamin D synthesis may also contribute [113]. Because PTH stimulates conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D, the functional hypoparathyroidism seen with severe hypomagnesemia may result in low circulating 1,25(OH)<sub>2</sub>D levels, further compromising the body's defense against hypocalcemia [114]. Hypomagnesemia can be seen in the settings of chronic gastrointestinal disease or nutritional deficiency, especially in alcoholics. To further complicate matters, vitamin D deficiency is often present in hypomagnesemic patients [115]. Replenishment of magnesium stores promptly and restores parathyroid function to normal.

Hypomagnesemia may result from inherited disorders of magnesium excretion and/or absorption [116] and can also be induced by the renal tubular effects of several drugs, including amphotericin B, aminoglycoside antibiotics, chemotherapeutic agents (particularly cis-platinum), diuretics, and cyclosporin. Primary disorders of magnesium wasting, due to variants in the *TRPM6* ion channel, claudin family members *CLDN16* (paracellin), *CLDN19*, and in the NaCl cotransporter *SLC12A3*, may have associated defects in renal tubular handling of calcium [116]. Thus, factors other than the effect of hypomagnesemia on PTH secretion/action may play a role in the hypocalcemia often accompanying this group of disorders.

## 5. Parathyroid hormone—-independent hypocalcemia

Despite normal PTH function and downstream signaling from its receptor, hypocalcemia can still occur due to disturbances in skeletal homeostasis, vitamin D metabolism, and a variety of medical illnesses.

### 5.1 Neonatal hypocalcemia

The newborn infant undergoes an acute transition to independently regulated mineral homeostasis at

parturition. When the maternal source of calcium is eliminated, the infant's circulating calcium level transiently decreases, with recovery occurring by the third day postpartum. Infants of diabetic or preeclamptic mothers and infants who suffer perinatal asphyxia or other fetal complications may experience an exaggerated fall in serum calcium with a delayed recovery phase. Management with intravenous calcium supplementation is required for symptomatic hypocalcemia or when severely low serum calcium levels are detected. This condition is referred to as "early neonatal hypocalcemia" and is usually transient. It may be associated with transient hypomagnesemia.

Hypocalcemia presenting at 5–10 days of life is referred to as "late neonatal hypocalcemia," occurring when the serum calcium is < 8 mg/dL in a term infant or < 7 mg/dL in a preterm infant (normal range in neonates and infants is approximately 9–11.3 mg/dL [117], significantly higher than older children and adults). Many of the genetic disorders of PTH deficiency, PTH resistance, hypomagnesemia, and vitamin D metabolism described elsewhere in this chapter present during this time. Reversible forms of neonatal hypocalcemia include increased phosphate load (see below), vitamin D deficiency (see later), hypomagnesemia (see before), citrate sequestration associated with blood transfusions, and alkalosis due to hyperventilation or bicarbonate therapy [75,117]. Mild to moderate neonatal hypocalcemia commonly occurs in patients with congenital heart disease [118] and in some cases can be attributed to transient impairment of parathyroid function.

### 5.2 Nutritional vitamin D deficiency

Vitamin D synthesis in the skin requires adequate exposure to ultraviolet light. Thus, vitamin D deficiency is less common in settings where sunlight exposure is abundant. In extremes of latitude (e.g., northern climates in North America) and where industrial pollution can interfere with transmission of UV light, normal vitamin D status is dependent on adequate dietary vitamin D intake. Supplementation of milk products with vitamin D has significantly reduced the incidence of vitamin D deficiency in North America. Despite these measures, certain populations are at significant risk for development of vitamin D deficiency, and severe hypocalcemia may be a presenting manifestation of the disorder (see Chapter 62 and Chapter 63).

A convergence of several risk factors for vitamin D deficiency occurs in breastfed infants during the first 18 months of life. Breastfed infants present with vitamin D deficiency most commonly during the winter or early spring in northern US cities. The limited direct sunlight

exposure during the winter season is a major factor. Individuals with darker skin are at greater risk due to the greater quanta of UV light required to penetrate the pigmented dermis and induce previtamin D formation [119]. Breast milk contains only small amounts of vitamin D, even when the mother is receiving pharmacological doses of the vitamin. Moreover, dietary practices, including vegetarian and high grain intake, may place infants at greater risk for the development of this condition [120]. Another group at high risk for development of vitamin D deficiency is found at the other extreme of life, the elderly because of general nutritional compromise and limited sunlight exposure.

Biochemical findings in these conditions vary with the severity or duration of deficiency. The circulating 25(OH)D level is generally used to assess total body vitamin D status; however, the definition of vitamin D deficiency is highly controversial. Serum calcium levels in moderate vitamin D deficiency are often normal, compensated by secondary elevations in PTH [121]. In severe vitamin D deficiency, however, overt hypocalcemia can occur, despite elevated circulating PTH. Serum phosphate levels tend to be slightly low or normal. Circulating alkaline phosphatase activity of bone origin is usually markedly elevated in children and can be elevated in adults. Bone symptoms (pain, leg-bowing in children) may persist after periods when 25(OH)D levels have been low, even while blood levels of 25(OH)D have normalized. In children, radiographs of rachitic extremities imaged after therapy has begun can reveal several distinctive features including hyperdense lines of remineralization at the physes, consistent with recent exposure to vitamin D, despite the continuing presence of physical findings of rickets. Circulating 1,25(OH)<sub>2</sub>D levels may be low, normal, or elevated during vitamin D deficiency. This may appear paradoxical, but it should be recognized that 1,25(OH)<sub>2</sub>D circulates in nearly 1000-fold lower concentrations than 25(OH)D. Furthermore, in the setting of vitamin D deficiency, production of 1,25(OH)<sub>2</sub>D is maximized. Thus, efficient conversion of small amounts of newly ingested or synthesized 25(OH)D may markedly increase the circulating 1,25(OH)<sub>2</sub>D concentration. An intriguing paradox in this setting is the continued malabsorption of calcium, despite normal concentrations of 1,25(OH)<sub>2</sub>D; recent data have shown that ambient reduction in calcium may suppress VDR expression, which may contribute to this phenomenon [122].

The skeletal consequence of isolated severe vitamin D deficiency in children is rickets, a disorder of the epiphyseal growth plate, characterized by the expansion of the hypertrophic zone of epiphyseal chondrocytes with subsequent disorganization and expansion of the growth plate cartilage matrix [123]. The defective mineralization processes ultimately result in deformities of the long bones. In adult bone, vitamin D deficiency causes

osteomalacia, which is characterized histomorphometrically by excess undermineralized osteoid and a markedly delayed mineralization rate. Adults with osteomalacia may suffer painful pseudofractures, particularly in weight-bearing long bones.

### 5.3 Vitamin D malabsorption

Because vitamin D is a fat-soluble vitamin, generalized fat malabsorption may result in vitamin D deficiency. Gastrointestinal disorders such as Crohn's disease, celiac disease, and pancreatic insufficiency can be accompanied by hypocalcemia due to vitamin D malabsorption [124]. In addition, interruption of the enterohepatic circulation of both 25(OH)D and 1,25(OH)<sub>2</sub>D may lower body vitamin D stores. The possibility that the diseased bowel may not be able to respond to 1,25(OH)<sub>2</sub>D may also contribute to calcium malabsorption in these settings. Mild hypocalcemia and secondary hyperparathyroidism are also seen in cholestatic liver diseases such as primary biliary cirrhosis. In the setting of low albumin, circulating levels of vitamin D–binding protein, total 25(OH)D, and free 25(OH)D are reduced in liver disease; 25(OH)D levels may not necessarily confirm adequate vitamin D status in liver disease [125]. It is not clear whether 25-hydroxylase (25-OHase) activity is significantly diminished in liver disease, although most reports suggest that the diseased liver retains the capacity for 25-hydroxylation when there is adequate substrate, except in situation of extreme liver failure [126]. Similarly, pathogenic variants in the gene encoding 25-OHase are a rarely reported cause of vitamin D–dependent rickets [127–130]. Liver transplantation typically improves vitamin D status, although immunosuppressive drugs may continue to have an adverse effect on calcium and vitamin D metabolism [131,132].

## 6. Genetic forms of vitamin D–dependent rickets

### 6.1 Deficiency of 1 $\alpha$ -OHase

Impaired metabolism of 25(OH)D to 1,25(OH)<sub>2</sub>D is characterized by hypocalcemia and severe rickets [130]. The disorder (also termed pseudo-vitamin D–deficient rickets or vitamin D–dependent rickets, type 1) is inherited in an autosomal recessive manner and is characterized by biochemical features similar to those of vitamin D–deficient rickets, with the exceptions that circulating 25(OH)D levels are normal and circulating 1,25(OH)<sub>2</sub>D levels are low (see Chapter 66). Loss-of-function variants in the gene encoding the ferredoxin-binding component of the mitochondrial P450 enzyme, 1 $\alpha$ -OHase (*CYP27B1*) or hepatic 25-hydroxylase (*CYP2R1B*), have been shown to cause

this condition [130,133]. Restoration of eucalcemia and correction of rickets are attainable with physiological doses of  $1,25(\text{OH})_2\text{D}$  [134].

## 6.2 Hereditary resistance to $1,25(\text{OH})_2\text{D}$

A defect in target-tissue responsiveness to  $1,25(\text{OH})_2\text{D}$  was clinically described shortly after the capacity to measure circulating  $1,25(\text{OH})_2\text{D}$  became available. Patients with hypocalcemia due to  $1,25(\text{OH})_2\text{D}$  resistance have severe manifestations of vitamin D-deficient rickets, despite normal serum  $25(\text{OH})\text{D}$  concentrations and elevated serum levels of  $1,25(\text{OH})_2\text{D}$ . This disorder is usually inherited in an autosomal recessive manner. Additional features in many patients include alopecia totalis and oligodontia [135].

The disease is variably responsive to large doses of  $1,25(\text{OH})_2\text{D}$  and oral calcium therapy. In the most resistant cases, long-term parenteral calcium infusions can normalize serum chemistries and cure the skeletal lesions [136]. The positive therapeutic response to parenteral calcium suggests that mediation of calcium absorption at the intestine is the critical systemic action for  $1,25(\text{OH})_2\text{D}$ .

There are several defects in the coding region of the VDR, which impair or prevent either hormone or DNA binding. Reduced levels of the VDR and variants in the gene encoding heterogenous nuclear ribonucleoprotein C (*HNRNPC*), a VDR coactivator, have also been described [137,138] (see Chapter 68).

## 6.3 Genetic causes of increased vitamin D metabolism

Recently an autosomal dominant form of genetic vitamin D deficiency rickets has been described; it is due to a gain-of-function variant *CYP3A4*. This encodes a P450 enzyme that metabolizes many xenobiotics and drugs, leading to vitamin D deficiency through accelerated vitamin D metabolite inactivation. The result of this variant is similar to adverse effects of drugs that are potent inducers of *CYP3A4* activity, such as rifampin, leading to vitamin D deficiency [139] (see Chapter 67).

## 7. Dietary calcium deficiency

Although uncommon, extremely low calcium intakes have been reported to be associated with mild hypocalcemia. Nigerian and South African children with calcium intakes of 150 mg/day or less were found to have reduced serum calcium values, secondary hyperparathyroidism, and rickets [140] without vitamin D

deficiency. Studies using stable calcium isotopes have demonstrated that intestinal calcium absorption in this group is not impaired [141] and children respond to treatment with calcium alone better than vitamin D alone, even when there is mild coincident vitamin D deficiency [142]. Similar findings have been observed in certain US populations. This phenomenon appears to occur after children have been weaned to diets with little to no dairy product content, with fluids consisting mostly of juices and soft drinks [143]. Thus, nutritional rickets may reflect calcium or vitamin D deficiency and often some combination of the two, given the common dairy source of these nutrients.

## 8. Hyperphosphatemia-mediated hypocalcemia

Since the 1930s, it has been appreciated that oral or parenteral phosphate can induce a decline in serum calcium concentrations. Hebert et al. have demonstrated that phosphate infusions lower serum calcium in both the presence and absence of parathyroid glands [144]. Moreover, they reported that the changes in peak urinary calcium excretion during phosphate administration are not sufficient to account for the fall in the serum calcium. The theory they advanced to explain this phenomenon centers on the hypothesis that the calcium  $\times$  phosphate molar product, when exceeded, leads to spontaneous precipitation of calcium salts in soft tissues. The biophysical mechanisms of soft tissue calcification are substantially more complex than invoked by this hypothesis or that can be detailed here. Nevertheless, clinical guidelines have been provided for use in patients with chronic kidney disease. While historically, the National Kidney Foundation KDOQI Guidelines recommended that the  $\text{Ca} \times \text{P}$  product be considered as a risk factor for extra-skeletal calcifications, more recent data suggest that this is not a well-validated marker [145,146].

Hyperphosphatemia sufficient to cause hypocalcemia is usually abrupt in onset and severe in magnitude. Typical clinical settings include (1) Excessive enteral or parenteral phosphate administration, (2) The tumor lysis syndrome, and (3) Rhabdomyolysis-induced acute renal failure. Hypocalcemia induced by phosphate administration is often associated with soft tissue calcification. Such ectopic calcification has been observed during the treatment of hypophosphatemia due to either diabetic ketoacidosis or acute alcoholism. Individuals receiving phosphate-containing enemas and infants fed “humanized” cow milk rich in phosphate may also become hypocalcemic [147,148]. Under most circumstances, discontinuation of exogenous phosphate intake leads to prompt return of the serum calcium level to normal.

Hypocalcemia in the setting of massive tumor lysis results from the release of intracellular phosphate



because of chemotherapy-induced cell death, usually during the treatment of rapidly proliferating neoplasms [149]. The hypocalcemia may continue beyond the period of hyperphosphatemia and appears to be aggravated by suppressed  $1,25(\text{OH})_2\text{D}$  levels [150]. The use of phosphate-binding antacids, oral calcium, and, in severe cases,  $1,25(\text{OH})_2\text{D}$  may help to correct the serum calcium level.

Rhabdomyolysis-induced acute renal failure occurs with trauma and drug or alcohol abuse. Marked hypocalcemia can occur in the early oliguric phase and moderate to severe hypercalcemia in the subsequent polyuric phase. Llach et al. have described hyperphosphatemia and suppressed serum  $1,25(\text{OH})_2\text{D}$  levels during the initial hypocalcemic phase, suggesting a mechanism similar to that seen in the tumor lysis syndrome [151]. The appearance of hypercalcemia and high serum  $1,25(\text{OH})_2\text{D}$  levels during the diuretic phase may result from rapid development of secondary hyperparathyroidism during the initial hypocalcemic period. Treatment includes restriction of phosphate intake and efforts to prevent hypocalcemia during the early stages of the disease.

## 9. Hypocalcemia due to accelerated skeletal mineralization

Bone remodeling is a controlled process of tissue renewal that, in healthy individuals, results in closely matched rates of bone resorption and formation. When skeletal mineralization exceeds the rate of bone resorption in the absence of sufficient ambient calcium, hypocalcemia can occur. One setting in which this can be observed is following surgical correction of primary or tertiary hyperparathyroidism. The abrupt cessation of PTH-mediated osteoclastic bone resorption with concomitant rapid remineralization of an under-mineralized skeleton can lead to “hungry bone syndrome” with severe, even life-threatening hypocalcemia [152]. Postoperative treatment should be instituted when the serum calcium level falls below 8.0 mg/dL, using oral or parenteral calcium supplements and calcitriol. In general, this condition resolves over the course of several days, although distinguishing it from permanent postoperative hypoparathyroidism can be difficult and requires gradual discontinuation of supportive therapy with careful monitoring. Patients with primary hyperparathyroidism who are taking cinacalcet should have their drug stopped several days before parathyroid surgery to reduce the risk of postoperative hypocalcemia. A phenomenon resembling the hungry bone syndrome has been observed when PTH 1–34 therapy is discontinued in hypo-parathyroid patients [153]. Hypocalcemia also may occur in patients with bony

metastases that induce bone formation, as with prostatic and breast cancer [154]. Finally, institution of therapy for vitamin D–deficient osteomalacia or rickets can sometimes lead to a fall in serum calcium associated with rapid mineralization of previously un-mineralized osteoid [155]. This is self-limited and can usually be prevented with supplemental calcium.

## 10. Medical illness

Several medical conditions are complicated by hypocalcemia. Decreased circulating calcium in the setting of renal failure results from hyperphosphatemia due to reduced renal phosphate clearance by the failing kidney and is complicated by impaired biosynthesis of  $1,25(\text{OH})_2\text{D}$  [156]. Hypocalcemia and tetany were first reported in patients with pancreatitis in the early 1940s [157]. Pancreatic lipase released from the damaged gland is believed to liberate free fatty acids that chelate calcium, thereby removing it from the extracellular fluid [158].

Hypomagnesemia resulting from poor oral intake, alcohol use, or vomiting may contribute to the hypocalcemia. Hypocalcemia in the setting of pancreatitis often suggests a poor clinical course [159]. In addition to increased skeletal mineralization, hypocalcemia in malignancy can be associated with numerous other factors, including cancer therapies and comorbid conditions [160].

Hypocalcemia may also occur in patients with acute sepsis [161]. In one series, 20% of such patients evidenced reductions in ionized serum calcium [162]. Hypocalcemia in this series was associated with a poor prognosis (50% mortality, compared with 30% in eucalcemic patients). This phenomenon is most often reported with gram-negative sepsis but has occurred in toxic shock syndrome caused by staphylococcal infection [163]. The hypocalcemia seen in meningococcal septicemia may be due to acute extravasation of calcium into the subcutaneous tissues [164]. Pro-inflammatory cytokines may also upregulate the CaSR, resulting in decreased PTH secretion and hypocalcemia [165].

Finally, it has been suggested that parathyroid gland reserve is subnormal in patients with AIDS, although hypocalcemia is not a prominent feature of that disorder [166].

### 10.1 Medications

A variety of medications have been reported to decrease serum-ionized calcium concentration. Many of these drugs are used to treat hypercalcemia and/or excessive bone resorption, and hypocalcemia results from their overzealous use. Thus, mithramycin, calcitonin, bisphosphonates, and denosumab can all cause hypocalcemia. In susceptible individuals, prolonged



therapy with diphenylhydantoin or phenobarbital can lead to hypocalcemia, owing in part to enhanced catabolism of vitamin D metabolites [167]. Citrated blood products, particularly when used for large-volume transfusions or plasma plasmapheresis, can cause hypocalcemia [168]. Radiocontrast agents that contain ethylenediaminetetraacetic acid can also induce falls in serum-ionized calcium levels [169]. Finally, foscarnet (trisodium phosphonoformate), used in the treatment of cytomegalovirus ocular infections and acyclovir-resistant herpes, has been reported to cause a decline in ionized serum calcium, perhaps through complexing extracellular calcium [170].

## 11. Treatment of hypocalcemia

### 11.1 Acute management

#### 11.1.1 Newborns and children

It may be necessary to treat early neonatal hypocalcemia. Appropriate emergency therapy of acute symptomatic hypocalcemia consists of a slow intravenous infusion of 10% calcium gluconate. One gram of 10% calcium gluconate is 1000 mg/10 mL; 9.3% of that is elemental calcium. Thus, there are 93 mg of elemental calcium in 10 mL of 10% calcium gluconate. For tetany or seizures, 1–3 mL can be given via slow intravenous push over 5–10 min with no more than 2 mg of elemental calcium per kilogram body weight given as a single dose. A well-functioning indwelling intravascular catheter should be used to avoid extravasation. Calcium should never be administered intramuscularly because of local tissue toxicity. It is important to perform cardiac monitoring and careful observation during acute infusions. Bolus infusions may be repeated at a slower rate up to 4 times in a 24 h period. If severe hypocalcemia persists, however, it is generally more effective to use a long-term calcium gluconate infusion, such that 20–50 mg of elemental calcium per kilogram body weight is infused over an entire 24 h period. Dosing for infants and older children is similar. Calcium chloride is more irritating than calcium gluconate and is not the preferred salt for infusion; it should never be administered through a peripheral vein. Neither bicarbonate nor phosphate should be co-infused with calcium to prevent precipitation of their respective calcium salts, either in the infusion line or in the vein.

#### 11.1.2 Adults

In adults, emergency management consists of 10–20 mL of 10% calcium gluconate infused over a 10–15 min period. In the longer term, one can dilute 100 mL of 10% calcium gluconate (representing 930 mg

of elemental calcium) in 1 L of 5% dextrose or normal saline and, beginning at a rate of 50 mL/h, titrate the rate to maintain the serum calcium in the low normal range. Typically, a dose of ~ 1.25 mg elemental calcium/kg body weight/hour is sufficient. As before, if only calcium chloride is available, a central line must be used for administration. It is important to monitor the patient's EKG during calcium infusions for adverse cardiac effects such as excessive shortening of the corrected QT interval or bradyarrhythmias. Finally, in the setting of acute exacerbations of calcium malabsorption, as may typically occur in patients with autoimmune hypoparathyroidism with associated gastrointestinal disorders, nocturnal nasogastric supplementation with calcium carbonate or calcium glubionate has been employed, providing up to 20 mg of elemental calcium per kilogram body weight per 8 h, as necessary, until the underlying intestinal disturbance has resolved.

#### 11.1.3 Role of magnesium supplementation

In the setting of hypomagnesemia, magnesium therapy may be required to restore PTH secretion and sensitivity of target tissues to the hormone. Prior to administration of magnesium salts, assessment of renal function and urinary output should be performed. Magnesium treatment in infancy consists of 5–10 mg of elemental magnesium per kilogram body weight. Although magnesium may be given intramuscularly, the intravenous route is preferred. Magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) is available as a 50% solution, containing 48 mg/mL of elemental magnesium. These small volumes may be further diluted, but they should be infused slowly; the dose may be repeated every 12–24 h. In older individuals, up to 2.4 mg of elemental Mg per kilogram body weight can be given over a 10 min period (to a maximum of 180 mg). Others prefer a continuous infusion of 576 mg of elemental magnesium over 24 h. The length of therapy must be individualized, and maintenance with oral magnesium salts should be implemented in cases where ongoing hypomagnesemia is anticipated.

Magnesium levels should be monitored to avoid toxicity. Deep tendon reflexes can be examined, and therapy should be halted if they diminish. As with calcium therapy, cardiac monitoring should be performed and therapy should be stopped if EKG changes occur. Intravenous calcium gluconate is a useful antidote for magnesium intoxication and should be available at the bedside.

### 11.2 Long-term management

Many of the causes of hypocalcemia discussed before are corrected by treating the underlying disorder (e.g.,

vitamin D deficiency, tumor lysis syndrome, hypomagnesemia disorders). Management of patients with chronic kidney disease is complex and varies depending on the degree of renal insufficiency; treatment may involve a combination of calcium, phosphate binders, vitamin D analogs, and calcimimetics [171]. Of those disorders requiring maintenance therapy for hypocalcemia, the most important are hypoparathyroidism, PHP, and inborn errors of vitamin D.

### 11.3 Hypoparathyroidism

In hypoparathyroidism, the distal tubule lacks the PTH signaling necessary for appropriate renal calcium reabsorption. Thus, serum calcium should be maintained at or slightly below the lower limit of the age-specific normal range to avoid hypercalciuria and nephrocalcinosis. Treatment should be titrated to reduce symptoms, such as circumoral tingling, paresthesia, and carpopedal spasm. Conventional therapy includes the combination of a vitamin D metabolite with calcium supplements, given in divided doses throughout the day [172]. A wide variety of preparations of both are available. The amount of elemental calcium varies based on preparation (Table 65.2). Because of the prolonged toxicity that occurs with excessive ingestion of calciferol, many prefer to use rapid-acting preparations of vitamin D (calcitriol, alfacalcidol), particularly in children. Toxicity, when it occurs with these latter preparations, corrects more rapidly with discontinuation of the drug. However, high doses of ergocalciferol or cholecalciferol can be used effectively, particularly in patients with poor compliance or concurrent mal-absorptive disorders, such as celiac disease or APECED. The dose of calcitriol typically ranges from as little as 0.25 up to 2.0 µg/day [173,174]. When higher doses are needed, calcitriol should be dosed twice daily because its biological half-life is approximately 12–14 h. Hypercalcemia, when it

develops during therapy with calcitriol, usually resolves within 3–4 days after discontinuing the drug.

High dietary calcium is generally recommended with the caveat that this should not come solely from dairy products, which also contain large amounts of phosphate. Phosphate-restrictive diets and phosphate binders are not routinely used. Most patients require additional calcium supplementation; 1000–2500 mg/day of elemental calcium in divided doses may be necessary. As enteral calcium supplements will reduce serum phosphate while vitamin D preparations will increase intestinal phosphate absorption, it is often preferable to first increase the calcium dose before adjusting the calcitriol dose. Commercially available calcium salts include carbonate, citrate, lactate, gluconate, and glucobionate. Calcium carbonate is 40% elemental calcium, inexpensive, well tolerated, and easily acquired. Patients are generally instructed to take it with food as gastric acidity appears to be required for absorption. However, the effects of acid-blocking medications on calcium absorption continue to be controversial [175,176]. Of note, many patients with hypoparathyroidism are also hypothyroid; as calcium decreases the absorption of levothyroxine, the timing of these medications should be separated. In some cases of hypoparathyroidism, a thiazide diuretic may be useful in augmenting serum calcium levels and reducing the hypercalciuria that can occur with the institution of treatment [177], although hypokalemia is an untoward side effect that should be closely monitored. However, the use of thiazides in hypoparathyroidism treatment has not been systematically tested. Likewise, a low-sodium diet may also reduce the risk of hypercalciuria, but this has not been well studied. There are many limitations of conventional therapy, specifically wide fluctuations in serum calcium, high pill burden, poor quality of life, and renal complications.

Magnesium deficiency can occur in patients with hypoparathyroidism, most often in patients with autosomal dominant hypocalcemia [178] or secondary to steatorrhea, which is seen in the autoimmune forms of this disorder [179,180]. This may render a patient relatively resistant to therapy, and therefore, magnesium deficiency should be considered in individuals whose therapeutic requirements unexpectedly increase. Symptoms of hypomagnesemia are similar to those of hypocalcemia, with some patient reporting decrease in symptoms when magnesium is added to their regimens.

PTH replacement therapy may improve the biochemical profile in those in whom conventional therapy is not adequate. Inadequate control may be due to symptomatic hypocalcemia, hyperphosphatemia, renal insufficiency, hypercalciuria, or poor quality of life. PTH replacement therapy for hypoparathyroidism has been under investigation since the mid-1990s, initially with the PTH 1–34 fragment, known as teriparatide and

**TABLE 65.2** Percentage of elemental calcium in different calcium salts.

	% Elemental calcium
Calcium acetate	25
Calcium carbonate	40
Calcium chloride	27
Calcium citrate	21
Calcium glucobionate	6.4
Calcium gluconate	9
Calcium lactate	13
Calcium phosphate, tribasic	39

more recently with intact PTH 1–84. Numerous studies have demonstrated that, in most pediatric and adult patients, the hypocalcemia can be adequately controlled with once or twice daily subcutaneous injections of PTH 1–34 or PTH 1–84, in some cases with patients receiving no supplemental calcium or calcitriol [181–183]. In a short-term placebo-controlled study of PTH 1–84, blood phosphate levels were also reduced, which may decrease the risk of extra-skeletal calcifications [184]. While most studies have failed to show persistent improvement in 24 h urine calcium excretion, a long-term open-label extension of this study did demonstrate reduction of mean 24 h urine calcium excretion into the normal range [185]. Of note, twice-daily PTH 1–34 induced significant hypo-citraturia, with 51% of patients developing new or worsening nephrocalcinosis or nephrolithiasis on imaging [186]. In the 6 year study by Rubin et al., 3 out of 33 subjects developed clinical nephrolithiasis in the final years of treatment [183].

In addition to the questionable effects on the kidney, the long-term skeletal effects of intermittent subcutaneous PTH therapy are unknown. Both PTH 1–34 and PTH 1–84 stimulate bone turnover dramatically, markedly increasing formation and resorption markers with persistent elevations maintained after several years of therapy [181,183,185,187]. Bone densitometry and iliac crest bone biopsies reveal the paradoxical skeletal actions of PTH therapy, with anabolic effects resulting in increased trabecular bone volume and catabolic effects evidenced as increased cortical porosity and decreased radial bone density [153,183,185,188]. Whether changes in bone with this exposure are transient is unknown; however, some data suggest that continued PTH therapy may restore the skeleton to normal [189,190]. Due to high bone turnover state induced, PTH therapy should not be abruptly discontinued but rather weaned slowly with concomitant administration of high doses of calcium and calcitriol to prevent severe, life-threatening hypocalcemia, similar to the hungry bone syndrome observed after parathyroidectomy in patients with hyperparathyroidism [153].

Subcutaneous recombinant full-length PTH 1–84 was approved in the United States in 2015 for the treatment of adults with hypoparathyroidism who cannot be adequately controlled with active vitamin D and calcium supplements [191] but was removed from the market in 2019 due to manufacturing issues. In November 2020, the US Food and Drug Administration (FDA) removed the “black box” warning indicating potential risk of osteosarcoma for teriparatide (PTH 1–34), which has been approved for osteoporosis but not hypoparathyroidism. While it is unclear whether intermittent subcutaneous PTH (1–34), teriparatide, is able to

prevent long-term complications in chronic hypoparathyroidism, replacement therapy with multiple daily injections of teriparatide appears to be effective at managing hypocalcemia for up to 10 years [192]. Recently, a phase 2, randomized, double-blind, placebo-controlled 4 week trial of TransCon PTH, an investigational long-acting prodrug of PTH(1–34), followed by an open-label extension, demonstrated significant reduction in oral active vitamin D and Ca requirements for most participants. Participants achieved normal serum calcium, serum phosphate, urine calcium, serum calcium-phosphate product, and improved health-related quality of life [193,194]. Small, short studies have suggested that continuous subcutaneous infusion of PTH may be more physiologic than intermittent injections [195,196]. Finally, development of new drugs including long-acting PTH analogs [197], PTH-related protein analogs, PTH receptor modulators [198], and allosteric modulators of CaSR [199] all hold promise that better therapies are yet to come.

Magnesium deficiency can occur in patients with hypoparathyroidism, most often in patients with autosomal dominant hypocalcemia [178] or secondary to steatorrhea, which is seen in the autoimmune forms of this disorder [179,180]. This may render a patient relatively resistant to therapy, and therefore, magnesium deficiency should be considered in individuals whose therapeutic requirements unexpectedly increase. Symptoms of hypomagnesemia are similar to those of hypocalcemia, with some patients reporting a decrease in symptoms when magnesium is added to their regimens.

### 11.4 Pseudohypoparathyroidism

In PHP, the medications used are the same, but the goals of therapy are different. The skeleton in patients with PHP is often responsive to the ambient hyperparathyroidism; thus, treatment is aimed at normalizing the PTH to decrease the risk of hyper-parathyroid bone disease, including osteoporosis and osteitis fibrosa cystica, as well as tertiary hyperparathyroidism [92,93,200]. This requires maintenance of the serum calcium level well within the normal range. As distal renal tubular calcium reabsorption remains responsive to PTH, hypercalciuria and nephrocalcinosis are extremely rare in these patients but can occur when overtreatment results in hypercalcemia and PTH suppression. Untreated patients with PHP may have varying degrees of osteopenia and initially may require high-dose therapy to achieve eucalcemia as their bones re-mineralize. Requirements will decrease as the bone heals, often heralded by a fall in serum alkaline phosphatase and a rise in serum calcium levels.

## 11.5 Genetic forms of vitamin D–dependent rickets

Individuals with 1 $\alpha$ -OHase deficiency have a defect in the ability to generate 1,25(OH)<sub>2</sub>D from the precursor metabolite 25(OH)D. In this disorder, eucalcemia can be achieved by supplying 1,25(OH)<sub>2</sub>D in physiological dosages [134]. In contrast, patients with hereditary resistance to 1,25(OH)<sub>2</sub>D due to pathogenic variants in the VDR can present with a spectrum of resistance to calcitriol therapy, with some individuals responding to doses of calcitriol in the usual therapeutic range and others resistant to even massive doses of the drug [138]. As noted before, chronic therapy with parenteral infusions of calcium has resulted in improvement of the rickets and normalization of all serum biochemical parameter [136]. Additionally, two novel analogs (20-epi-1,25(OH)<sub>2</sub>D and JK-1626–22) have been found to be somewhat more efficacious than 1,25(OH)<sub>2</sub>D itself in the treatment of cases of hereditary resistance to vitamin D caused by pathogenic variants in the ligand-binding domain of vitamin D receptor [201].

Even with excellent patient adherence to the treatment regimen and careful monitoring, a variety of stresses including trauma, immobilization [202], infection, gastrointestinal illness, pregnancy, and lactation [203] can increase or decrease the therapeutic requirements. Thus, extra vigilance is required during those times. Providing patients with a standing laboratory order for a calcium measurement allows them to obtain blood work at the first sign of symptoms, enabling the practitioner to adjust medication doses rapidly and ideally avoiding hospitalization for a hypo or hypercalcemic crisis.

## 12. Conclusion

Hypocalcemia can be due to numerous genetic and acquired conditions, primarily related to alterations in PTH and vitamin D metabolism. Identifying the underlying etiology of hypocalcemia is important as therapeutic options and treatment goals vary depending on the cause. In the case of heritable conditions, genetic counseling of affected individuals is critical to anticipate and avoid hypo-calcemic seizures in their children, particularly newborns. Although calcium and traditional vitamin D analogs remain the mainstay of therapy for most chronic hypocalcemic conditions, newer, directed therapies are in development that will hopefully allow for precision management of patients, while avoiding the morbidities associated with conventional therapies.

## 13. Summary points

- Regulatory mechanisms maintain calcium within a narrow range by calciotropic hormones, PTH, and vitamin D.
- Hypocalcemia can be due to numerous genetic and acquired conditions, primarily related to alterations in PTH and vitamin D metabolism.
- Hypocalcemic disorders are classified by their functional etiologies and discussed in relationship to primary homeostatic disturbances including inadequate PTH secretion, resistance to PTH action, or PTH independence.
- Identifying the underlying etiology of hypocalcemia is important as therapeutic options and treatment goals vary depending on the cause.

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# Vitamin D hydroxylation—deficient rickets, type 1A

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## OBJECTIVES

- Present the main clinical manifestations and biochemical changes in patients with VDDR1A.
- Explain the molecular etiology of the disease and the mutations affecting patients.
- Discuss treatment alternatives and the accepted standard of care.
- Describe the impact of treatment over the course of the life of the patients.

## 1. Introduction

The term “rickets” is erroneously used to describe all of the skeletal abnormalities associated with defective mineralization in the growing skeleton, but it is more precise to restrict the term to changes in the growth plate and adjacent metaphysis. When mineralization is impaired, the accumulation of unmineralized osteoid at sites other than the growing metaphysis should be referred to as osteomalacia, not as rickets. Thus, defective mineralization can lead to both rickets and osteomalacia in the growing skeleton but only to osteomalacia in the mature skeleton.

Rickets is characterized by the inadequate calcification of the growth plate and adjacent metaphysis. The impaired mineralization of the growth plate cartilage in the zone of provisional calcification prevents this zone from being resorbed (see Chapter 62 and Chapter 63). As the cartilage continues to be formed, but not

resorbed, the growth plate begins to widen. Simultaneously, the trabecular bone directly underneath the cartilage fails to mineralize properly, and vascularization of this tissue becomes aberrant. These defects are accompanied by similar abnormalities in cortical bone, leading to the full spectrum of skeletal symptoms associated with the pathology.

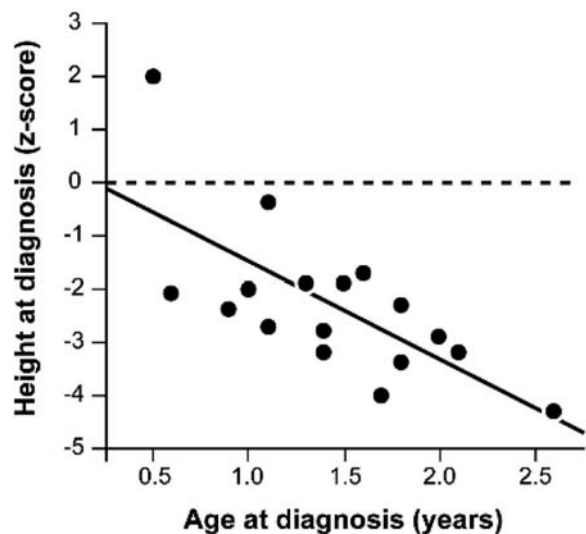
“Rickets resistant to vitamin D therapy” were described clinically by Albright et al. in 1937 [1]. Subsequent reports [2,3] suggested a variant of resistant rickets that was fully clinically described by Prader et al. in 1961 [4]. The absence of knowledge of the underlying molecular etiology of these diseases led to several, somewhat confusing designations: pseudo-vitamin D deficiency rickets (PDDR) [4]; vitamin D dependency (VDD) [5]; vitamin D-dependent rickets, type I (VDDR-I); or 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase) deficiency [6]. In 1997, remarkable progress was made in the understanding of the molecular etiology of the disease through the identification of mutations in the gene encoding the 25-1 $\alpha$ -OHase (*CYP27B1*) in affected patients, leading to hydroxylation defects and the inability to synthesize 1,25(OH)<sub>2</sub>D [7]. For this reason, and to align with current OMIM (Online Mendelian Inheritance in Man compendium) nomenclature, we will use the OMIM designation of vitamin D hydroxylation—deficient rickets, type 1A (VDDR1A, OMIM 264700) to describe the disease.

Other genetic defects of vitamin D metabolism and action with related nomenclature include vitamin D hydroxylation—deficient rickets, type 1B (VDDR1B, OMIM 600081) (Chapter 67), caused by mutations in the *CYP2R1* gene [8–11] encoding the microsomal vitamin D 25-hydroxylase [12] and involving a defect



**TABLE 66.1** Affected genes and clinical manifestations in VDDR 1B, 1A, and 2A.

Feature	VDDR1B	VDDR1A	VDDR2A
Mutations	<i>CYP2R1</i>	<i>CYP27B1</i>	<i>VDR</i>
Genetic inheritance	Autosomal recessive	Autosomal recessive	Autosomal recessive
Age of onset	Late (4–5 years of age)	Early	Early
Rickets	Yes	Yes	Yes
Hypocalcemia	Yes	yes	Yes
Serum alkaline phosphatase	Elevated	Elevated	Elevated
Secondary hyperparathyroidism	Yes	Yes	Yes
Alopecia	No	No	Yes
Serum 25(OH)D	Low to undetectable	Normal	Normal
Serum 1,25(OH) <sub>2</sub> D	Normal/high	Low	Elevated
Response to 25(OH)D therapy	Yes	No	No
Response to 1,25(OH) <sub>2</sub> D therapy	Partial	Yes	No

**FIGURE 66.1** Height z-score according to age at time of diagnosis in young children with VDDR1A. From Ref. [14] with permission.

in vitamin D 25-hydroxylation. Vitamin D–dependent rickets type 2A (VDDR2A, OMIM 277440) (Chapter 68) is caused by mutations in the vitamin D receptor (*VDR*) gene [13]. This condition is also referred to as hereditary vitamin D resistant rickets (HVDRR). Although clinically similar, VDDR1A is characterized by extremely low to absent serum 1,25(OH)<sub>2</sub>D levels, while VDDR2A is characterized by very high levels of circulating 1,25(OH)<sub>2</sub>D (Table 66.1).

## 2. Clinical manifestations

Patients with VDDR1A are healthy at birth, and the first symptoms usually appear within the first year of life. The majority of patients (85%) present with neurological signs: delayed gross motor development and hypotonia (70%), or hypocalcemic seizures (15%). Growth retardation is a common manifestation (70%), and height at the time of diagnosis is reduced for age (Fig. 66.1). Pathological fractures may occur. In the past, when the diagnosis was either missed or made too late to intervene, infant death by hypocalcemia or pulmonary infections was frequent. A history of adequate mineral and vitamin D intake, without evidence of intestinal malabsorption, is a constant finding.

Physical examination reveals a short, hypotonic child with features of rickets. There is a wide anterior fontanel with frontal bossing and frequent craniotables (easy depression of the softened parietooccipital area). Tooth eruption is delayed, and erupted teeth show evidence of enamel hypoplasia. A “rachitic rosary” (enlargement of the costochondral junctions along the anterolateral area of the chest) is either visible or palpable. The development of a Harrison sulcus (depression on both sides of the chest wall between the pectoral muscles and the lower margin of the ribcage) caused by the muscular pull of the diaphragmatic attachments to the lower ribs can be observed. In the appendicular skeleton, enlargement of the metaphyseal areas is more evident in the wrists and ankles, and there is a variable degree

of deformity (bowing) of long bone diaphyses. The site and type of deformity of the extremities depend upon the age of the child and the weight-bearing patterns in the limbs. Thus, deformities of the forearms and anterior bowing of the distal tibia are found more commonly in infants, whereas an exaggeration of the normal physiological bowing of the legs (*genu varum*) is a characteristic finding in the VDDR1A toddler who has started to walk. The Chvostek sign (twitching of the upper lip on light finger tapping of the facial nerve) reflects nerve irritability, a consequence of a rapid drop in serum calcium.

Radiological examination of the skeleton reveals diffuse osteopenia and the classic metaphyseal changes of vitamin D deficiency. There is a fraying, cupping, widening, and fuzziness of the zone of provisional calcification immediately under the growth plate. These changes are seen better and detected earlier in the most active growth plates, namely, the distal ulna and femur and the proximal and distal tibia. Changes in the diaphyses may not be evident when metaphyseal changes are first detected. However, they will appear a few weeks later as rarefaction, coarse trabeculation, cortical thinning, and subperiosteal erosion. The latter reflects the increased resorption induced by secondary hyperparathyroidism. When performed, bone mineral density (BMD) of the lumbar spine is very low [14].

### 3. Biochemical findings

Hypocalcemia is the cardinal feature in VDDR1A. Serum calcium concentration will drop below 2 mmol/L (8 mg/dL). This, particularly if the decrease is rapid, may give rise to tetany and convulsions, which may occur prior to any radiological evidence of rickets. Persistent hypocalcemia triggers secondary hyperparathyroidism and hyperaminoaciduria [15]. Urinary calcium content is low, whereas fecal calcium is high, reflecting impaired intestinal calcium absorption.

Serum phosphate concentration may be normal or low. Hypophosphatemia, when present, is usually of a lesser degree than in X-linked hypophosphatemia. It is the result of both impairment of intestinal absorption and increased urinary loss induced by secondary hyperparathyroidism. Serum alkaline phosphatase activity is consistently elevated (over 800 IU/L). Its increase often precedes the appearance of clinical symptoms. The calcemic response to PTH is usually, but not necessarily, absent [16].

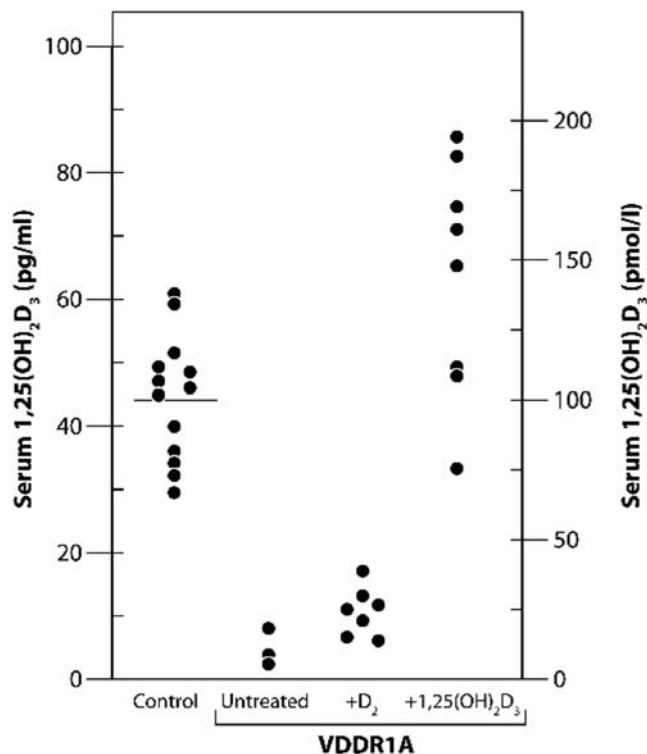
Studies of circulating vitamin D metabolites have provided a key insight into the pathogenesis of VDDR1A. Serum levels of 25(OH)D are normal in the untreated patient. These results indicate that intestinal absorption of vitamin D and its hydroxylation in the liver are not

impaired in VDDR1A. On the contrary, circulating levels of 1,25(OH)<sub>2</sub>D are low [16–18]. This is evident rapidly after birth, months before any clinical or radiological signs of rickets develop. Even when patients are treated with large doses of vitamin D, causing major increases in the circulating levels of 25(OH)D, 1,25(OH)<sub>2</sub>D levels do not reach the normal range (Fig. 66.2). This clearly identifies defective activity of the 1 $\alpha$ -OHase as the basic abnormality in VDDR1A and differentiates it from vitamin D-dependent rickets type 2A (Table 66.1). Although 1,25(OH)<sub>2</sub>D serum levels are low, in many cases they remain detectable and positively correlated to the serum concentration of 25(OH)D [17].

Circulating levels of 24,25(OH)<sub>2</sub>D are normal in VDDR1A patients and are highly correlated with those of 25(OH)D, indicating a fully functional vitamin D 24-hydroxylase (CYP24A1) enzyme [19].

### 4. Genetic and molecular studies

Animal models of VDDR1A were generated independently by three laboratories using targeted inactivation of the gene of interest in mice [20–22]. The phenotypic consequences of *Cyp27b1* inactivation in mouse preclinical models are detailed in Chapter 30.



**FIGURE 66.2** Serum 1,25(OH)<sub>2</sub>D concentrations in control children and in VDDR1A patients either untreated or treated with high doses of vitamin D or calcitriol.

VDDR1A is inherited as a simple autosomal recessive trait [5]. No phenotypic abnormalities have been observed in heterozygous carriers of mutations [23]. Although generally rare, VDDR1A may be more common in genetically isolated populations as a result of founder effects. For example, VDDR1A occurs at an unusually high frequency in the French Canadian population originating from the Charlevoix-Saguenay-Lac Saint Jean region of the province of Quebec [24,25]. This high local incidence of 1 in 2400 live births with a carrier rate of 1 in 26 [26] allowed mapping of the disease gene to chromosome 12q14 [27]. Microsatellite haplotyping identified distinct founder populations between the Charlevoix-Saguenay-Lac Saint Jean area and the eastern Canadian (Acadian) region [28]. Founder effects have also been reported in patients from Korea [29].

The human CYP27B1 enzyme is encoded by a single gene of nine exons and eight introns that is 5 kb in length [30]. The ferredoxin-binding domain is encoded by sequences contained in exons 6 and 7, while the heme-binding domain is contained in exon 8 [30,31].

At the time of updating of this chapter, 83 distinct CYP27B1 mutations causing VDDR1A had been reported (Table 66.2). Identified disease-causing sequence changes include missense and nonsense mutations, deletions, duplications, and splice mutations [7,14,23,29,32,34,35,38–45,47–54]. These mutations are dispersed throughout the CYP27B1 gene, affecting all exons and five introns (Table 66.2). There are a fair number of compound heterozygous mutations (different mutation on each CYP27B1 allele) in affected patients. A frequent mutated allele is the 7bp insertion (CCCACCC) in exon 8 (c.1325–1332ins7), present in several unrelated ethnic groups [23,32,34,35,41]. This mutation alters the downstream reading frame, causing a premature stop codon 16bp downstream. One patient was also reported with a 9bp insertion at the same position (c.1325\_1334ins9), again causing a frame shift and further suggesting that this area is a mutational hot spot [34].

CYP27B1 structure models based on other cytochrome P450 proteins have some credibility, such as the three-dimensional model of human CYP27B1 based on the crystal structure of the rabbit microsomal P450 enzyme, CYP2C5 [58]. This model allowed prediction of the outcome of several mutations causing VDDR1A [59]. Similarly, linear alignment of P450 enzyme sequences permits logical deductions concerning the impact of VDDR1A disease-causing mutations on CYP27B1 catalytic activity [34,60].

A number of assays have also been used to study the enzymatic activity of the missense CYP27B1 mutants, including expression of the recombinant protein in heterologous cells that express the electron carriers

adrenodoxin reductase and adrenodoxin [34], activation of vitamin D receptor–dependent gene transcription [45,49], or enzymatic activity in peripheral blood mononuclear cells [23,48]. In the few cases where missense CYP27B1 mutant proteins retained residual capacity to hydroxylate 25(OH)D on carbon 1 (assayed by expressing the mutant cDNA in heterozygous cells), the VDDR1A patients exhibited mild clinical biochemistry changes [39,43]. However, most of the mutant proteins tested did not retain enzymatic activity, a surprising finding considering that residual amounts of 1,25(OH)<sub>2</sub>D were measured in the serum of patients harboring the studied mutations [49]. Whether this reflects spurious 1 $\alpha$ -hydroxylation of 25(OH)D by other cytochrome P450 proteins when CYP27B1 is defective or detection of food-derived 1,25(OH)<sub>2</sub>D is unclear [49].

Thus, establishing genotype–phenotype correlations has been challenging in VDDR1A. An interesting approach using correlation between CYP27B1 mutations, age at presentation, height standard deviation score and calcitriol doses required for treatment has been reported [61]. Using these metrics, it was possible to establish statistically significant genotype–phenotype correlations among the three most prevalent mutations within the Turkish population. Of clinical severity (milder to more severe), the mutations were ranked as p.K182E < p.F443Pfs\*24 (the 7bp duplication) < c.195+2T > A (intron 1) [61]. It could prove useful to compare the height, age at presentation, and calcitriol dose of treatment for all mutations to determine the validity of the genotype–phenotype correlation across all cases reported.

## 5. Treatment of VDDR1A

Historically, patients with VDDR1A were treated with high doses of vitamin D (calciferol, 20,000 to 100,000 IU/day), in an attempt to overcome CYP27B1 deficiency, with a certain success [15,62]. Under such treatment, circulating levels of 25(OH)D increase sharply, with only minor changes in the levels of 1,25(OH)<sub>2</sub>D (Fig. 66.2). It is likely that massive concentrations of 25(OH)D are able to overcome its low affinity for the VDR to adequately bind to the VDR and act as an agonist to induce the response of the target organs to normalize calcium homeostasis. However, because such therapy leads to progressive accumulation of vitamin D in fat and muscle tissues, adjustment in case of overdose is difficult and slow to come into effect. Furthermore, the therapeutic doses are close to the toxic doses and place the patient at risk for nephrocalcinosis and impaired renal function. There have been reports on the use of 25(OH)D as therapeutic agent in VDDR1A [63]. The doses used are smaller than those of vitamin D

**TABLE 66.2** Mutations in CYP27B1 reported up to February 2022.

Nucleotide change <sup>a</sup>	Change	Exon	References
c.48_60del13	p.E20Pfs*2	1	[32]
c.85G > T	p.E29*	1	[33]
c.165delG	p.K55fs*22	1	[34]
c.170G > T	p.G57V	1	[32]
c.171delG	p.L58Cfs*20	1	[35]
c.171dupG	p.L58Afs*275	1	[36]
c.195G > T	p.Q65H	1	[34]
c.195+2T > A	—	Intron 1	[37]
c.195+2T > G	—	Intron 1	[38]
c.201_204delinsCTTCG	p.Q67Hfs*89	2	[39]
c.217G > T	p.G73W	2	[32]
c.217G > C	p.G73R	2	[14]
c.242G > A	p.G81E	2	[40]
c.258delG	p.Y87Tfs*24	2	[34,41]
c.262delG	p.V88Wfs*24	2	[14]
c.286_300del15	p.E95delELLRQ	2	[42]
c.305G > A	p.G102E	2	[43]
c.311_321del11	p.R104Lfs*225	2	[44]
c.320G > A	p.R107H	2	[45]
c.335C > T	p.P112L	2	[29]
c.374G > A	p.G125E	2	[45]
c.386+1G > A	—	Intron 2	[39]
c.398_400dupAAT	p.W134*	3	[46]
c.403C > T	p.Q135*	3	[47]
c.403delC	p.Q135Kfs*8	3	[14]
c.413G > T	p.R138L	3	[48]
c.428C > T	p.P143L	3	[49]
c.473T > C	p.L158P	3	[44]
c.490G > A	p.D164N	3	[49]
c.565G > A	p.E189K	3	[34]
c.566A > G	p.E189G	3	[49]
c.574A > G	p.K192E	3	[35]
c.580G > A	p.G194R	3	[33]
c.580G > T	p.G194*	3	[33]
c.590+1G > A	—	intron3	[49]
c.590G > A	p.G197D	4	[35]
c.626delG	p.C209Sfs*9	4	[7]

Continued

**TABLE 66.2** Mutations in CYP27B1 reported up to February 2022.—cont'd

Nucleotide change <sup>a</sup>	Change	Exon	References
c.692delC	p.T232Rfs*1	4	[7]
c.723G > A	p.W241*	4	[34]
c.934_935delAC	p.T312Rfs*6	5	[50]
c.962C > G	p.T321R	5	[49]
c.968C > A	p.S323Y	6	[23]
c.983G > A	p.W328*	6	[29]
c.997C > T	p.L333F	6	[32]
c.1004G > C	p.R335P	6	[45]
c.1022-1037del16	p.T341Rfs*2	6	[50]
c.1027C > T	p.L343F	6	[39]
c.1079C > A	p.S360*	6	[38]
c.1136+1G > T	—	Intron 6	[51]
c.1136+2T > A	—	Intron 6	[50]
c.1144C > T	p.P382S	7	[34]
c.1144C > G	p.P382A	7	[40]
c.1165C > T	p.R389C	7	[52]
c.1165C > G	p.R389G	7	[49]
c.1166G > A	p.R389H	7	[34]
c.1215+1G > A	—	Intron 7	[29]
c.1215+2T > A	—	Intron 7	[37]
c.1226C > T	p.T409I	8	[34]
c.1232G > A	p.C411Y	8	[48]
c.1238A > G	p.Y413C	8	[14]
c.1286G > C	p.R429P	8	[34]
c.1294C > T	p.R432C	8	[32]
c.1294C > A	p.R432S	8	[53]
c.1299G > A	p.W433*	8	[49]
c.1310delG	p.G437Vfs*12	8	[32]
c.1319–1325dup7	p.F443Pfs*24	8	[37]
c.1321C > T	p.H441Y	8	[54]
c.1325–1332ins7	p.H441fs*4	8	[34]
c.1325–1334ins9	p.H441fs	8	[34]
c.1357C > T	p.R453C	8	[29]
c.1358G > A	p.R453H	8	[14]
c.1375C > T	p.R459C	8	[32]
c.1376G > T	p.R459L	8	[54]
c.1433T > G	p.V478G	9	[23]

Continued



**TABLE 66.2** Mutations in *CYP27B1* reported up to February 2022.—cont'd

Nucleotide change <sup>a</sup>	Change	Exon	References
c.1442delA	p.E481Gfs*179	9	[33]
c.1446delA	p.G483Vfs*19	9	[32]
c.1474C > T	p.R492W	9	[32]
c.1475G > C	p.R492P	9	[14]
c.1490C > G	p.P497R	9	[34]
c.1494delA	p.R499Gfs*14	9	[29]
c.1504delA	p.N502Tfs*141	9	[33]
c.1510C > T	p.Q504*	9	[55]
c.1521delC	p.D507Efs*34	9	[56]

<sup>a</sup>Nucleotide numbers refer to coding sequence (CDS; NCBI consensus CDS database CCDS8954.1) and are numbered from the translation start site. Amino acid replacement mutations are indicated according to codon number. Nomenclature follows standard mutation nomenclature in molecular diagnostics [57]. Reference indicates first report.

and induce a similar response. The action of 25(OH)D is likely to be similar to that of vitamin D itself, by maintaining high serum concentrations of 25(OH)D. The low availability and high cost of such a preparation have discouraged its widespread use as a long-term therapy for VDDR1A. Treatment with the synthetic analog of 1,25(OH)<sub>2</sub>D, dihydrotachysterol, has also been used [64,65] but is currently discontinued.

Clinicians have used the monohydroxylated analog 1 $\alpha$ -hydroxyvitamin D (1 $\alpha$ OHD), which requires only liver hydroxylation at the 25 position (a step not affected by the VDDR1A mutation) to fully mimic 1,25(OH)<sub>2</sub>D [66]. The response is rapid with healing of rickets in 7–9 weeks, requiring a daily dosage of 2–5  $\mu$ g. The maintenance dose is about half the initial dose. Withdrawal induces a reappearance of symptoms within 3 weeks. Thus, long-term compliance is a more important consideration than in the case of vitamin D treatment. On a weight basis, 1 $\alpha$ (OH)D is about half as potent as 1,25(OH)<sub>2</sub>D, nullifying any possible economic advantage in favor of the monohydroxylated form. The reason for this difference in potency has not been investigated, but may be related to a difference in intestinal absorption or to a variable degree of 25-hydroxylation of 1 $\alpha$ (OH)D.

Since calcitriol (1,25(OH)<sub>2</sub>D<sub>3</sub>; Rocaltrol) became commercially available in 1973, the treatment of choice for VDDR1A patients is a long-term (lifelong) replacement therapy with calcitriol [17]. This results in rapid and complete correction of the abnormal phenotype, eliminating hypocalcemia, secondary hyperparathyroidism, and radiographic evidence of rickets. Strikingly, the myopathy disappears within days after initiation of therapy. The restoration of bone mineral

content is equally rapid, and histological evidence of healing of the bone structure has been observed [17].

Treatment with calcitriol (in capsules containing either 0.25 or 0.5  $\mu$ g) is started at a dose of 1.0  $\mu$ g per day, given in two doses of 0.5  $\mu$ g. Subsequently, the calcitriol dose is modified according to the results of biochemical analyses. The aims of the treatment are to achieve normocalcemia, to maintain PTH levels within normal limits and to avoid hypercalciuria. In our experience, the median daily calcitriol dose is 0.50  $\mu$ g per day (range: 0.2–1.0  $\mu$ g) after 3 months of treatment, 0.25  $\mu$ g after 1 year (range 0.1–1.0  $\mu$ g), and 0.5  $\mu$ g after 2 years (range 0.25–1.0  $\mu$ g) (personal data). An important component of treatment is to ensure adequate calcium intake during the bone-healing phase. Dietary sources are supplemented to ensure a daily supply of around 1 g of elemental calcium.

## 6. Evolution of VDDR1A under treatment from childhood to adulthood

### 6.1 Short-term effects of treatment with calcitriol

Replacement therapy with calcitriol results in rapid and complete correction of the abnormal phenotype, eliminating hypocalcemia, secondary hyperparathyroidism (Fig. 66.3), and radiographic evidence of rickets within 3 months (Fig. 66.4) [17].

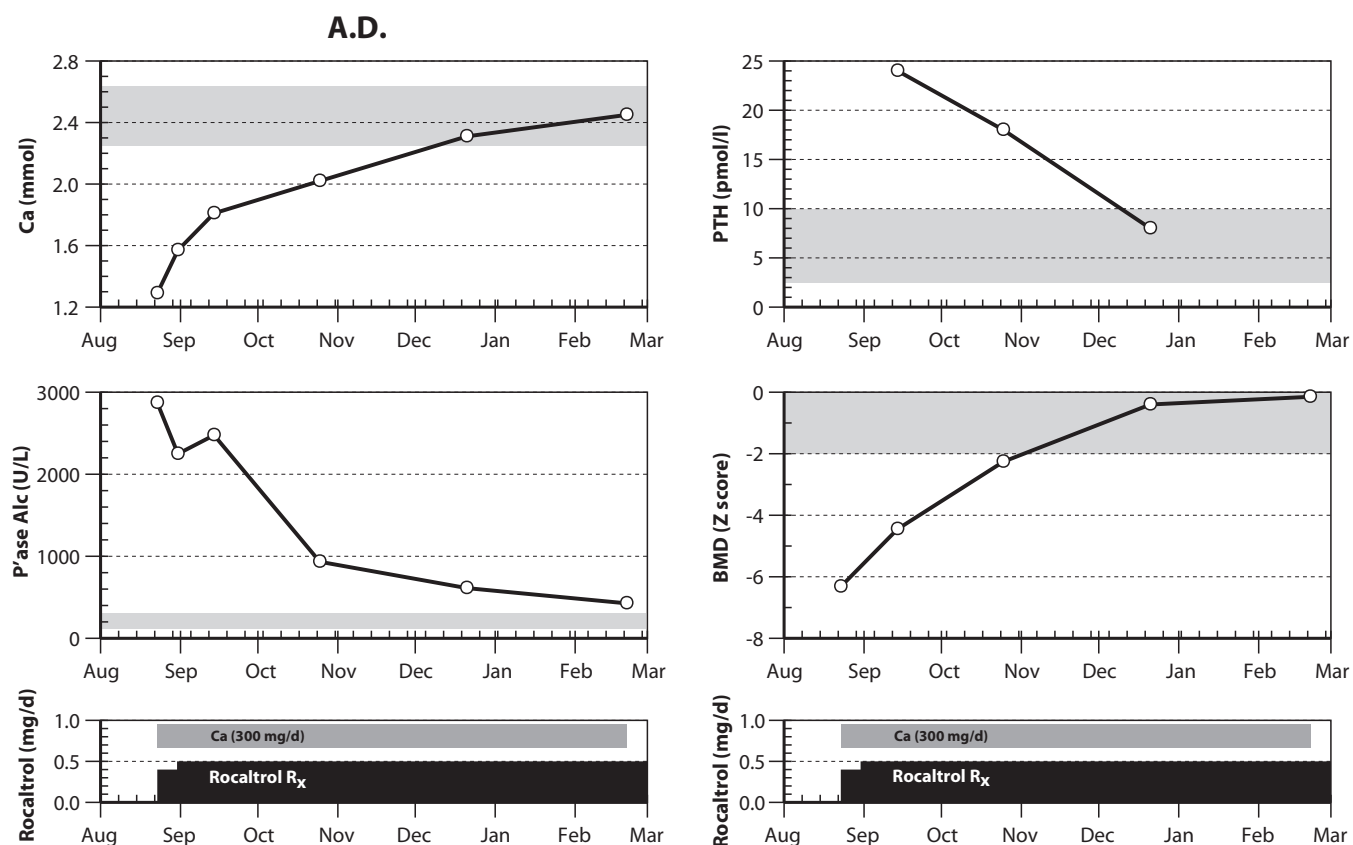
Lumbar spine areal BMD also normalizes within 3 months (Fig. 66.3) [14], as described in children and adults who are treated for vitamin D deficiency [67]. The rapidity of the increase in BMD suggests that calcitriol treatment of VDDR1A patients initially leads to the mineralization of preexisting unmineralized osteoid rather than the production of new bone matrix. Histological evidence of healing has been documented in VDDR1A patients [17] as well as in the animal model of VDDR1A [68].

The height deficit persists somewhat longer than the low areal BMD, but after 2 years of calcitriol treatment, height is also normalized (catch-up growth) and remains so until growth is completed (Fig. 66.5) [14].

Severe enamel hypoplasia is only partially corrected if treatment, as is usually the case, is started around 12–15 months of age when permanent tooth enamel has already started to develop (Fig. 66.6).

### 6.2 From childhood to adulthood

From childhood to adulthood, calcitriol doses are increased as needed to maintain serum and urinary calcium as well as PTH levels within normal limits. In our experience, in the period from 4 to 9 years of age, the



**FIGURE 66.3** Biochemical response to treatment in a patient with VDDR1A treated with calcitriol.

median daily calcitriol dose increases from 0.25 to 0.50  $\mu\text{g}$ . From 11 to 15 years of age, during the pubertal growth spurt, median calcitriol doses increase from 0.50  $\mu\text{g}$  per day to 0.75  $\mu\text{g}$  per day and remain so until adulthood [14]. The increased dose is recommended to avoid hypocalcemia and secondary hyperparathyroidism. It has been proposed that the urinary calcium to creatinine ratio, when less than 0.1 mg/mg in VDDR1A adolescent patients, is a useful biomarker to accurately evaluate calcium depletion and secondary hyperparathyroidism [69].

### 6.3 Adult patients with VDDR1A

Adult height is significantly associated with the age at which calcitriol treatment was started (Fig. 66.7) [14].

The height of adult patients who receive calcitriol before the pubertal growth spurt is normal, whereas patients who receive calcitriol only after the pubertal growth spurt are significantly shorter (Table 66.3). Lumbar spine areal BMD is normal in all adult patients whatever the treatment history. With regard to serum levels of vitamin D metabolites under calcitriol treatment, 25(OH)D levels and 1,25(OH)<sub>2</sub>D are normal in most patients.

Hypercalciuria is not infrequent during treatment with calcitriol, and changes in urinary calcium excretion are also used to adjust the daily calcitriol dose. High levels of calcium excretion may lead to calcium deposition in tissues especially in cornea and kidneys. In our treatment protocol, renal ultrasound and slit-lamp examinations of the cornea are performed every 2 years to assess for the presence of nephrocalcinosis and corneal calcium deposits. This screening for potential adverse events of hypercalcemia revealed that one patient (4% of the study population of 25) had mild corneal calcium deposits and four patients (16%) had mild nephrocalcinosis on renal ultrasound albeit with no alteration of renal function. None of our 47 patients treated with calcitriol for up to 40 years has evidence of reduced renal function (based on annual creatinine clearance evaluation). Kidney stones have never been observed (personal communication).

### 6.4 Pregnancies in women with VDDR1A

Female mice that are deficient for *Cyp27b1* (and that do not receive calcitriol) have uterine hypoplasia, absent corpora lutea, and thus are infertile [21]. This defect is at least partly due to hypocalcemia. Moreover, the

*CYP27B1* gene is expressed in human endometrial stromal cells independent of the cycle phase but with a significant increase in early pregnant decidua, suggesting a potential role of local production of  $1,25(\text{OH})_2\text{D}$  in pregnancy establishment or maintenance [70,71]. The study of decidual tissues from two *VDDR1A* patients showed

that it did not have the capacity to produce  $1,25(\text{OH})_2\text{D}$ , indicating that decidua is a target for the *VDDR1A* mutation [72]. However, the physiological importance of this defect is unclear.

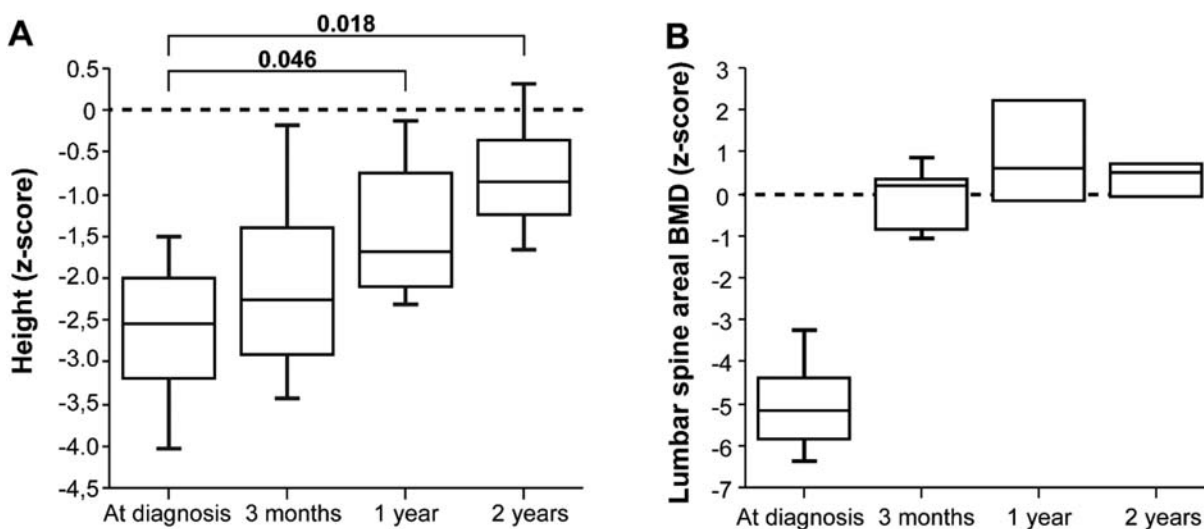
In our experience, pubertal development seemed to be normal in treated *VDDR1A* children. In our cohort, 9 of 13 women with *VDDR1A* who were above 20 years of age have had 19 documented pregnancies [14]. It therefore appears that local production of  $1,25(\text{OH})_2\text{D}$  in female reproductive organs is not critical for fertility, as systemic supplementation with calcitriol and normalization of serum calcium was sufficient to achieve fertility. During normal pregnancy,  $1,25(\text{OH})_2\text{D}$  circulating levels steadily increase to about twice the control values. This adaptation to the specific needs of pregnancy can be mimicked in pregnant women with *VDDR1A* by increasing the daily calcitriol dose during



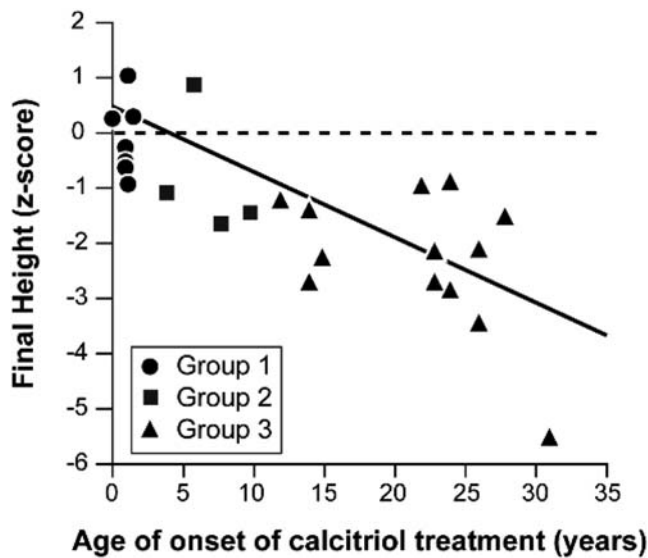
**FIGURE 66.4** Radiographs of the right wrist (*upper panel*) and knee (*lower panel*) of a patient with *VDDR1A*, before treatment (*left panel*) and after only 3 weeks of treatment (*right panel*); healing of rickets is well underway.



**FIGURE 66.6** Permanent incisors of a 9-year-old patient with *VDDR1A* in whom calcitriol treatment was initiated at age 14 months. The part of the enamel that was formed before treatment remains hypoplastic. Subsequent to treatment, normal enamel was produced.



**FIGURE 66.5** Evolution of height (in 12 patients) and areal BMD of the lumbar spine (in five patients) during the first 2 years of treatment with calcitriol in young children with *VDDR1A*. From Ref. [14] with permission.



**FIGURE 66.7** Final height according to age of onset of calcitriol treatment in adult patients with VDDR1A. Group 1, exclusively treated with calcitriol. Group 2, initially treated with high doses of vitamin D but started calcitriol before the pubertal growth spurt. Group 3, initially treated with high doses of vitamin D but started calcitriol after the pubertal growth spurt. From Ref. [14] with permission.

the second half of pregnancy. During pregnancy, doses of calcitriol are adjusted according to the results of biochemical analyses (obtained every 4 weeks) to maintain serum calcium levels within normal limits. After delivery, calcitriol treatment is returned to prepregnancy doses. All pregnancies were without complications. In particular, there was no case of intrauterine growth retardation, and all newborns were normocalcemic at birth.

## 7. Conclusion

Vitamin D hydroxylation-deficient rickets, type 1A (VDDR1A) is a rare autosomal recessive disorder caused by mutations in the gene encoding  $1\alpha$ -OHase (CYP27B1), leading to an inability to synthesize  $1,25(\text{OH})_2\text{D}$ . This disease was the first described inborn error of vitamin D metabolism and has contributed in a major way to our understanding of vitamin D biology. The absence of any other overt phenotype besides symptoms related to hypocalcemia supports the widely held view that the major physiological role of the

**TABLE 66.3** Auxological, biochemical, and radiological data of adult patients with VDDR1A.

	Group 1		Group 2		Group 3		P
	N	Value (range)	N	Value (range)	N	Value (range)	
Number (female/male)	7	7 (1/6)	4	4 (4/0)	14	14 (9/5)	
Age (years)	7	20.0 (17.0; 270)	4	20.2 (18.0; 26.0)	14	31.0 (24.0; 45.0) <sup>a,b</sup>	0.0009
Age at treatment onset	7	1.0 (0.1; 1.6)	4	7.0 (4.0; 10.0)	14	23.0 (12.0; 31.0) <sup>a,b</sup>	0.0001
Duration of treatment (years)	7	19.1 (15.2; 26.2)	4	14.8 (11.2; 18.2)	14	11.0 (3.5; 24.1) <sup>a</sup>	0.0025
Dose of calcitriol ( $\mu\text{g/day}$ )	7	0.75 (0.75; 1.00)	3	1.25 (0.50; 1.50)	14	0.5 (0.50; 1.00)	0.24
Height (z-score)	7	-0.3 (-0.9; 1.0)	4	-1.3 (-1.6; 0.9)	14	-2.2 (-5.5; -0.9) <sup>a</sup>	0.0008
Weight (z-score)	7	0.5 (-1.4; 1.4)	4	1.0 (0.43; 2.18)	14	-1.3 (-3.0; 1.4) <sup>a,b</sup>	0.011
Total calcium (mmol/L) (Norm: 2.25-2.63)	7	2.37 (2.29; 2.50)	4	2.33 (2.26; 2.55)	12	2.32 (2.10; 2.45)	0.39
Phosphate (mmol/L) (Norm: 1.23-1.62)	7	1.24 (0.87; 1.36)	4	1.07 (0.75; 1.22)	12	0.87 (0.74; 1.13) <sup>a</sup>	0.008
Alkaline phosphatase (U/l) (Norm: <300)	7	86 (47; 130)	4	85 (39; 158)	12	61 (35; 103)	0.36
PTH (% normal maximum)	7	82 (42; 166)	3	90 (50; 90)	11	60 (50; 150)	0.99
Urinary calcium/creatinine	7	0.4 (0.1; 0.7)	4	0.3 (0.1; 0.6)	11	0.5 (0.1; 1.1)	0.50
LS volumetric BMD (z-score)	7	-1.1 (-1.9; 2.7)	3	0.9 (0.0; 30)	14	0.4 (-0.9; 2.3)	0.42

<sup>a</sup>Significantly different between groups 1 and 3.

<sup>b</sup>Significantly different between groups 2 and 3.

P values calculated using the Kruskal-Wallis test.

Group 1: exclusively treated with calcitriol.

Group 2: initially treated with high doses of vitamin D; started calcitriol before the pubertal growth spurt.

Group 3: initially treated with high doses of vitamin D; started calcitriol after the pubertal growth spurt.



1,25(OH)<sub>2</sub>D hormone is to maintain mineral homeostasis.

The main site for the 1-hydroxylation of 25(OH)D is the proximal tubule of the renal cortex [73]. It is well known, however, that *CYP27B1* is expressed in extrarenal tissues such as osteoblasts [74,75], chondrocytes [76–78], keratinocytes [7], and cells of the lymphohematopoietic system [79,80] (see Chapter 9). The identification of these extrarenal sites of expression of *CYP27B1* has led investigators to hypothesize that local production of 1,25(OH)<sub>2</sub>D could play an important intracrine, autocrine, or paracrine role in the differentiation or function of the cells expressing *CYP27B1*. For example, activation of Toll-like receptors in human macrophages, a component of innate immune responses, leads to upregulation of the expression of the *VDR* and *CYP27B1*. This provokes the intracrine vitamin D–dependent induction of the antimicrobial peptide cathelicidin and increases the microbicidal activity of the macrophages [79] (see Chapter 94). These results demonstrate the physiological relevance of extrarenal *CYP27B1* expression in vitamin D–mediated innate immunity.

Molecular genetic studies have also revealed direct, but nonessential, roles for 1,25(OH)<sub>2</sub>D-mediated signaling in growth plate chondrocytes. Taken together, the results from genetic manipulation of the expression of *Cyp27b1* [81] or the *VDR* [82] in chondrocytes show a direct role for locally synthesized 1,25(OH)<sub>2</sub>D<sub>3</sub>, acting through the *VDR*, in vascular invasion and osteoclastogenesis during endochondral bone development, causing a transient increase in bone volume at the primary spongiosa [81,82].

1 $\alpha$ -OHase is expressed by both osteoblasts and osteocytes. Experiments using osteoblastic cell lines suggest that intracrine/paracrine production of 1,25(OH)<sub>2</sub>D by osteoblasts enhances their differentiation and bone mineral deposition [83–85]. Mice deficient for *Cyp27b1* specifically in osteoblasts are normocalcemic and normophosphatemic and show normal levels of circulating 1,25(OH)<sub>2</sub>D. However, conditional osteoblastic deletion of *Cyp27b1* decreased steady-state serum level of fibroblast growth factor 23 (FGF23), and local synthesis of 1,25(OH)<sub>2</sub>D in osteoblasts appears particularly important for FGF23 expression in kidney disease [86]. When the *Cyp27b1* gene was specifically inactivated in osteocytes in a model of chronic kidney disease, soft-tissue ectopic calcification was significantly increased [87], suggesting that enhanced osteocytic 1,25(OH)<sub>2</sub>D production acts as a protective mechanism against pathological soft-tissue mineralization in uremia.

1 $\alpha$ -OHase is present in many tissues in normal individuals but not expressed in the tissues of VDDR1A patients that were tested (skin, decidua, peripheral blood mononuclear cells) [7,23,72]. It is important to point

out that replacement therapy with calcitriol in VDDR1A patients leads to rapid and long-term rescue of the abnormal phenotype. Treated subjects are in general good health, fertile, and free of immunological problems. Thus, normalization of the systemic concentration of 1,25(OH)<sub>2</sub>D is necessary and sufficient to nullify the effects of the *CYP27B1* mutations. It ensures that despite all the interesting observations pointing to possible important autocrine/paracrine roles of 1,25(OH)<sub>2</sub>D in various tissues, their importance in human physiology remains to be established.

## 8. Summary points

- VDDR1A (OMIM 264700): Vitamin D hydroxylation—deficient rickets, type 1A now designates mutations in the gene encoding the 1 $\alpha$ -OHase (*CYP27B1*) and replaces previous nomenclature for the disease (PDDR, VDD, VDDR-1, or 1 $\alpha$ -OHase deficiency).
- VDDR1A is inherited as a simple autosomal recessive trait; heterozygotes are not affected.
- Generally rare, VDDR1A may be more common in genetically isolated populations as a result of founder effects.
- The treatment of choice is lifelong replacement therapy with 1,25(OH)<sub>2</sub>D<sub>3</sub>.

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# The role of genetic variation in CYP2R1, the principal vitamin D 25-hydroxylase, and CYP3A4 in vitamin D homeostasis

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## OBJECTIVES

- Review the data supporting the role of CYP2R1 as the principal vitamin D 25-hydroxylase.
- Discuss the identification of *CYP2R1* as the cause for VDDR-1B.
- Discuss the effect of a rare gain-of-function mutation in CYP3A4 to cause VDDR-3.
- Review work analyzing in vitro activity effects and predicted structural effects of mutations on CYP2R1.
- Review evidence supporting the model that regulation of CYP2R1 contributes to trends in 25(OH)D observed in aging, asthma, and obesity.

## 1. Introduction

Normal vitamin D homeostasis is necessary for optimal bone and mineral metabolism and may also play an important role in modulating neurologic, immune, and cardiovascular function. For most humans, the principle source of parent vitamin D is sunlight-induced production of cholecalciferol (vitamin D<sub>3</sub>) by the skin. Ultraviolet irradiation (wavelengths between 290 and 315 nm) of keratinocytes of the stratum basale and stratum spinosum layers of the epidermis leads to

conversion of 7-dehydrocholesterol to previtamin D<sub>3</sub>, which thermally isomerizes to form vitamin D<sub>3</sub>. Vitamin D<sub>3</sub> is subsequently transported into the circulation via vitamin D-binding protein (DBP). With excessive UVB exposure, previtamin D<sub>3</sub> can also isomerize to form the inactive photoproducts tachysterol or lumisterol, which do not enter the circulation and apparently represent alternative products that prevent vitamin D toxicity. Vitamin D can also be obtained through the diet as either cholecalciferol or ergocalciferol (vitamin D<sub>2</sub>) [1]. The parent calciferols are biologically inactive, and they must undergo two hydroxylations to become fully active [2]. The first hydroxylation is performed principally in the liver by the enzyme CYP2R1, which generates 25-hydroxyvitamin D (calcidiol; 25(OH)D) [3]. A second hydroxylation is required to produce calcitriol (1,25(OH)<sub>2</sub>D), the fully active form of vitamin D. This transformation occurs principally in renal proximal tubule cells, where substrate 25(OH)D undergoes 1 $\alpha$  hydroxylation by CYP27B1 in a tightly regulated reaction that is stimulated by parathyroid hormone (PTH) and inhibited by the phosphatonin fibroblast growth factor 23 [4]. Calcitriol is degraded to inactive forms primarily by CYP24A1, but it can also be metabolized to inactive forms by CYP3A4 [5]. Although calcitriol is the hormonally active form of vitamin D, 25(OH)D is considered the most useful indicator of vitamin D status as it is the most abundant circulating form of vitamin D [6].

Vitamin D deficiency (a serum concentration of 25(OH)D less than 20 ng/mL or 50 nmol/L) and

insufficiency (a serum concentration of 25(OH)D less than 30 ng/mL or 75 nmol/L) are common conditions throughout the world, and they occur even in countries near the equator where sunlight exposure and UVB irradiation are abundant year round. One mechanism to explain lower circulating levels of 25(OH)D among people living in these regions of the world is decreased cutaneous synthesis of vitamin D<sub>3</sub> because of darker skin pigment and/or more conservative styles of dress. However, there is also emerging evidence to suggest that genetic variations in *CYP2R1* may also play an important role in pathogenesis of vitamin D insufficiency in these (and other) populations [7].

## 2. CYP2R1 is the principal human vitamin D 25-hydroxylase

The primary site of 25(OH)D production from parent vitamin D is the liver [8]. Within the liver, 25-hydroxylase activity has been identified *ex vivo* in both microsome-enriched fractions and mitochondrial fractions [9]. At least six hepatic P450 enzymes possess vitamin D 25-hydroxylase activity *in vitro* (Table 67.1): rat CYP2C11, porcine CYP2D25, rat CYP2J3, CYP27A1, CYP3A4, and CYP2R1 [10–18]. With the exception of CYP3A4, which is located in the mitochondria, all of these enzymes are associated with the microsomes. The human functional homolog of rat CYP2C11, CYP2C9, is expressed at lesser abundance in the human liver relative to CYP2C11 expression in the rat liver, and it is therefore unlikely to be responsible for significant 25-hydroxylation in humans. Similarly, the functional human ortholog of porcine CYP2D25, CYP2D6, is significantly decreased in abundance relative to CYP2D25 expression in the pig liver, and it has no detectable 25-hydroxylase activity *in vitro*. Additionally, human CYP2J2 has tenfold less activity than its rat ortholog, CYP2J3, making it unlikely to represent a significant source of 25-hydroxylation in humans.

In humans, CYP3A4 and CYP27A1 can also function as vitamin D 25-hydroxylases, but each appears to have a different primary role. CYP3A4 is expressed in the intestine and the liver, and it is the predominant hepatic P450 enzyme responsible for oxidative metabolism of a wide variety of small molecules and xenobiotics, including an estimated 60% of all clinically used drugs. CYP27A1 can 25-hydroxylate vitamin D, but it lacks specificity, and it also hydroxylates the C-26 and C-27 positions. Moreover, vitamin D metabolism and 25(OH)D levels are normal or elevated in humans [19] and mice [20] that lack functional CYP27A1, and mutations in CYP27A1 are associated with cerebrotendinous xanthomatosis [21].

By contrast, genetic studies in knockout mice in which *Cyp2r1* has been ablated [20,22], and humans with spontaneous mutations of *CYP2R1* [3,23] provide compelling evidence that CYP2R1 is the principal 25-hydroxylase for vitamin D metabolism, although it is unclear what proportion of 25-hydroxylase activity is because of CYP2R1 in mammals [20].

The *CYP2R1* gene (OMIM: 608713) is located on human chromosome 11p15.2 and contains five exons that encode a 501-amino-acid protein with a molecular mass of 57,359 Da. *CYP2R1* has homologs in all mammalian genomes that have been sequenced, and its amino acid sequence is 80% conserved across these species. In the mouse and the rat, there appear to be two predominant splice variants, but the full-length transcript is significantly more abundant than the shorter forms. The functional significance of these variants as well as the relative translation efficiency and enzymatic activity of the CYP2R1 proteins produced from these smaller transcripts has not been characterized.

CYP2R1 localizes to the microsomal membranes and has been proposed to homodimerize during gel filtration based on its apparent migration size (120 kDa) [24], though it is not clear if this homodimer exists *in vivo*. CYP2R1 coordinates a heme cofactor, and for catalytic activity, it accepts electrons from the NADPH-cytochrome P450 oxoreductase protein. Similar to other CYP-P450 enzymes, the crystal structure of CYP2R1 has a fold consisting of  $\alpha$ -helices (A–L) with  $\beta$ -sheets mostly on one side of the molecule and with the heme buried deep inside the protein (Figs. 67.1A and 67.2—heme in red [stick representation]). The core of the protein is formed by alpha helices (E, I, J, K, and L). In CYP2R1, the F–G loop contains two small helices F' and G'; this latter helix (G') is highly hydrophobic, and for another similar CYP-P450 enzyme, CYP2C9, it has been hypothesized to be associated with the endoplasmic reticular membrane. The crystal structure identifies residues (highlighted in green in Fig. 67.1A) from four helices and four loops that form the vitamin D-binding pocket (the B' helix: Leu114, Phe115, Met118, from the B–C loop: Leu125, Asn126, from the F-helix: Phe214, Asn217, Ala221, from the G helix: Ala250, Val253, Tyr254, from the I helix: Phe302, Glu306, Ala310, Thr314; from the loop between helix K and  $\beta$ -sheet 1: Val375, Ile379, and from the loop between C-terminal  $\beta$ -sheet 4: Met487, Thr488). Heme coordination relies on hydrogen bonding between the seven residues (highlighted in orange in Fig. 67.1A) orange Trp133, Arg137, Arg446, Arg109, His381, and Ser442).

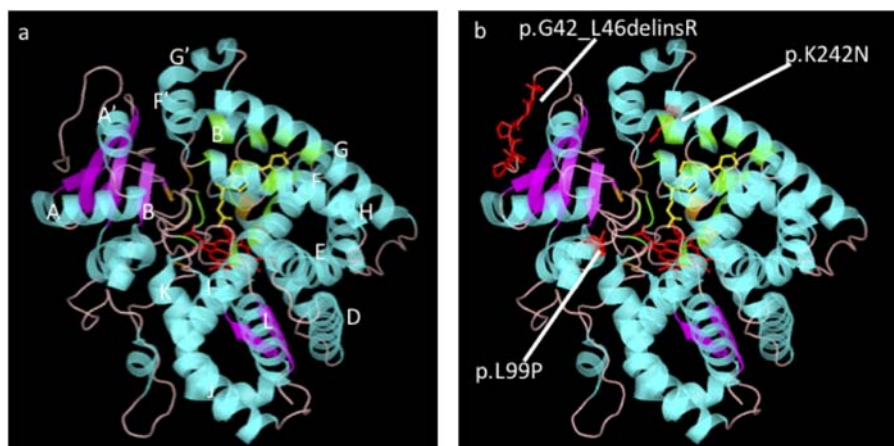
Emerging understanding of CYP2R1 has stimulated great interest in this enzyme for at least three important reasons. First, CYP2R1 is highly conserved across all examined mammalian species, which implies an

**TABLE 67.1** Enzymes that have vitamin D 25-hydroxylase activity in vitro.

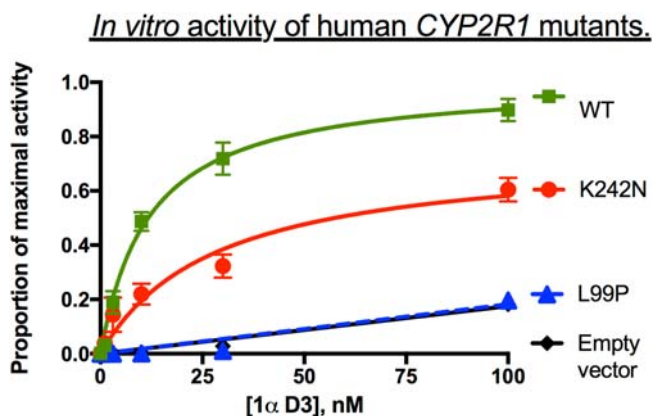
Enzyme (reference)	Human functional homolog	Highly expressed organs	Primary subcellular location	Preferred substrate	OMIM	Enzyme commission number (EC) for human enzyme)	Notes
Rat CYP2C11 [10]	CYP2C9	Liver	Microsomes	D3	601130	1.14.13.48	Human variants linked to poor warfarin metabolism
Porcine CYP2D25 [11]	CYP2D6	Liver	Microsomes	D2/D3	124030	1.14.14.1	Most polymorphic human CYP; human variants linked to codeine sensitivity
Rat CYP2J3 [12]	CYP2J2	Liver	Microsomes	D2 > D3	N/A	1.14.14.1	Human 25-hydroxylase activity is 10-fold less than rat functional homolog
Human CYP3A4 [15]	—	Liver, intestine, CNS, thyroid	Microsomes	D2	124010	1.14.13.97	Most abundant human CYP.
Human CYP27A1 [13]	—	Liver, lung, monocyte lineage	Mitochondria	D3	606530	1.14.13.15	Mouse knockout leads to increased serum 25(OH)D; human null variants cause cerebrotendinous xanthomatosis
Human CYP2R1 [18]	—	Liver and intestine	Microsomes	D2/D3	608713	1.14.13.159	Associated in GWAS at genome-wide significance ( $<10^{-8}$ ) with serum 25(OH)D

CNS, central nervous system; CYP, cytochrome P450; GWASs, genome-wide association studies.





**FIGURE 67.1** Overall structure of human CYP2R1. Distal view of CYP2R1 structure with  $\alpha$ -helices in light blue and  $\beta$ -strands in light pink. (A) All helices are labeled. The heme is in red (stick representation). Vitamin D3 is yellow. Residues believed to be important in the vitamin D-binding site are highlighted in green (residues from the B' helix: Leu114, Phe115, Met118, from the B-C loop: Leu125, Asn126, from the F-helix: Phe214, Asn217, Ala221, from the G helix: Ala250, Val253, Tyr254, from the I helix: Phe302, Glu306, Ala310, Thr314; from the loop between helix K and  $\beta$ -sheet 1: Val375, Ile379, and from the loop between C-terminal  $\beta$ -sheet 4: Met487, Thr488). Residues believed to be important in heme coordination are in orange (Trp133, Arg137, Arg446, Arg109, His381 and Ser442). (B) Discrete mutants clinically described (p.G42\_L46delinsR, p.L99P, and p.K242 N) are labeled with those residues visible in red (stick representation).



**FIGURE 67.2** Response of normal and mutant CYP2R1 enzymes to increasing concentrations of 1 $\alpha$ -hydroxyvitamin D3. *GAL4*, galactose 4; *UAS*, upstream activator sequence; *VDR*, vitamin D receptor. The expression plasmids were introduced into HEK293 cells with DNAs constituting the *VDR-GAL4/GAL4-UAS-luciferase* 1-hybrid reporter gene system. Relative activity is expressed as the ratio of induced firefly luciferase to control (constitutive) Renilla luciferase. Points on the graphs represent means of triplicate values established at each concentration of secosteroid.

important biological role. Investigation of CYP2R1 is thus broadly significant and clinically relevant especially given the fundamental significance of P450 enzymes to metabolism of hormones and drugs. Second, expression of CYP2R1 in nonhepatic tissues challenges previously held notions of vitamin D metabolism. Although CYP2R1 is highly expressed in the liver, recent analyses have shown that this enzyme is also expressed at significant levels in the testis [25–29], which raises important questions about the role of locally produced

metabolites of vitamin D in the testis, and the contribution of the testis to systemic vitamin D metabolism. For example, testicular CYP2R1 is reduced in patients with idiopathic testicular failure and correlates with lower circulating levels of 25(OH)D and increased serum PTH [25]. And perhaps not unexpectedly, vitamin D has reciprocal effects on testicular function and fertility: irrespective of cause, reduced circulating levels of 25(OH)D are associated with decreased sperm quality [30,31], and sperm motility is stimulated by calcitriol in vitro [32]. The production of CYP2R1 transcripts by spermatogonia supports the possibility that high local concentrations of 25(OH)D, or calcitriol, may be critical for normal maturation of spermatogonia. Vitamin D receptor (VDR) KO mice have altered expression of estrogen receptor subtypes in spermatogonia [33] as well as elevated gonadotropin concentrations [34]. Moreover, low serum levels of 25(OH)D in patients with Klinefelter syndrome [35] may reflect another consequence of testicular dysfunction, and it has been proposed by some that reduced serum levels of 25(OH)D may be as or more important than low circulating levels of testosterone as an explanation for low bone density and osteoporosis in these patients [36].

Third, and as described in greater detail in the following, genome-wide association studies (GWAS) have identified variants in or near the *CYP2R1* locus, which are associated with serum concentrations of 25(OH)D, providing evidence that genetic factors as well as environment and diet influence production of 25(OH)D. Moreover, Mendelian randomization studies implicate these same variants as predisposing to complications of obesity, multiple sclerosis, and overall

mortality. Therefore, understanding how variants effect CYP2R1 activity and predispose to disease may guide development of novel approaches for prevention and/or treatment of these disorders.

### 3. CYP2R1 and vitamin D-dependent rickets

Mutations in *CYP2R1* that reduce expression or function of the CYP2R1 enzyme have been associated with rickets and low circulating levels of 25(OH)D, with reduced responsiveness to vitamin D supplementation [3,23,37–40]. This condition is now considered a unique form of vitamin D-dependent rickets (VDDR) termed type VDDR-1B (OMIM #600081). Subjects described in these reports require increased vitamin D supplementation or calcium supplementation to treat their clinical rickets (Table 67.2) and show decreased responses to vitamin D supplementation that are gene dose-dependent (Table 67.3).

The initial description of what has come to be known as VDDR-1B was published in 1994 [37]. Two brothers of Nigerian origin who were living in Baltimore presented with rickets and very low serum concentrations of 25(OH)D that did not respond appropriately to therapeutic doses of vitamin D. It was not until a decade later that the genetic basis for this unusual form of vitamin D resistance was identified as homozygous missense mutations (p.L99P) in *CYP2R1* [3]. Functional studies revealed that recombinant CYP2R1 enzyme carrying the p.L99P mutation had negligible 25-hydroxylase activity compared with wild-type recombinant CYP2R1 whether the substrate was vitamin D<sub>2</sub> or vitamin D<sub>3</sub>. These observations provided evidence that loss-of-function mutations in *CYP2R1* are a novel cause of VDDR and, second, that CYP2R1 is the primary vitamin D 25-hydroxylase in humans.

Subsequent work described the vitamin D pathophysiology in individuals homozygous for the p.L99P mutation but residing in Nigeria [38]. The subject had clinical rickets and very low serum 25(OH)D, despite extensive sun exposure. The subject who was homozygous for the p.L99P mutation had low calcium, low phosphorous, elevated serum 1,25(OH)D<sub>2</sub>, and also undetectable 25(OH)D. By contrast to other forms of VDDR, however, a relative who was heterozygous for the p.L99P mutation had a decreased basal 25(OH)D concentration reflecting a pathophysiologic effect of haploinsufficiency. Interestingly, this work also described greater responses to large doses of cholecalciferol relative to ergocalciferol in both homozygotes and heterozygotes for the p.L99P mutation, perhaps reflecting the possibility that this mutant, or other enzymes with 25-hydroxylase activity, can hydroxylate cholecalciferol better than ergocalciferol.

A later report of VDDR-1B described compound heterozygous mutations in an affected member of a family of Middle Eastern descent [39]. One of these mutations is an insertion and frameshift mutant (c.768\_769insT (p.L257SfsX6)), and the other is a change in the splice donor site at the end of exon two (c.367 + 1, G → A). The insertion results in a frameshift and the deletion of amino acids involved in forming the binding site for vitamin D, whereas the splice site mutation removes a splice site that is present in all of the reported splice variants for *CYP2R1* mRNA. Both of these changes are predicted to result in nonfunctional proteins; however, no molecular or functional studies performed to confirm these predictions have been published. Absent such studies, it is not clear whether the loss of the donor site leads to production of a protein that includes sequences encoded by retained intronic DNA, a protein missing amino acids in a skipped exon, or no protein production at all from this allele. The female index case described in this paper was able to increase her serum 25(OH)D concentration into the normal range with high-dose vitamin D supplementation consisting of 5000 IU cholecalciferol per day, which might indicate a partial deficiency of CYP2R1 rather than an absolute deficiency, or as previously speculated, the contribution of alternative 25-hydroxylase enzymes. By contrast, an affected male sibling was not able to increase his 25(OH)D into the normal range despite supplementation with 10,000 IU of cholecalciferol per day, which was attributed to poor compliance. An alternative explanation for the discrepant 25(OH)D response to vitamin D of the two affected siblings is unbalanced transcription from the two alleles, assuming one mutation is more destructive than the other, or possibly sex-dependent dimorphism for CYP2R1 activity, or for another 25-hydroxylase. No studies were performed in the heterozygous relatives.

More recent work [23] described in greater detail the pathophysiology of subjects from Nigeria with VDDR-1B who were homozygous for the p.L99P mutation from two families, as well several of as their heterozygous relatives. In addition, a subject heterozygous for a novel missense mutation, p.K242N, was also described. As in the initial report, basal serum levels of 25(OH)D were lower in both homozygotes and heterozygotes. In addition, subjects who were homozygous for the p.L99P mutation showed negligible 25(OH)D responses to oral administration of vitamin D<sub>2</sub> or vitamin D<sub>3</sub>, whereas subjects who were heterozygotes for the p.L99P and p.K242N mutation had greater responses that were still subnormal consistent with a pathophysiologic effect of haploinsufficiency.

Finally, the most recent report characterizing individuals with VDDR-1B describes two unrelated families in France [40]. One family had several individuals who

**TABLE 67.2** Biochemical and clinical features of subjects with *CYP2R1* mutations.

Publication	Subjects	Mutation	Serum calcium (2.18–2.58 mmol/L)	Serum phosphorus (adults: 0.8 –1.5 mmol/L; children: 1.3 –2.3 mmol/L)	Serum alk phos (adults: 38–126 U/ L; children: Age and gender dependent, generally 90 –500 U/L)	Serum 25(OH)D (>50 nmol/L)	Serum 1,25(OH)2D (58–156 pmol/ L)	Serum PTH (11 –54 pg/mL; 1.2 –5.8 pmol/L)	Clinical response to supplementation
Casella et al. and Cheng et al. [3,37]	7-year-old male	Homozygous for <i>p.L99P</i>	2 mmol/L	0.84 mmol/L	2360 U/L	16 nmol/L	142 pmol/L		Required 3000 IU/ day of D2 for normalization
	2-year-old male	Homozygous for <i>p.L99P</i>	2.32 mmol/L	0.87 mmol/L	3000 U/L	12 nmol/L	137 pmol/L	697 pg/mL	Required 4000 IU/ day of D2 for normalization
Al Mutair et al. [39]	13-year-old female	c.768_769insT ( <i>p.L257SfsX6</i> ) c.367 + 1 G → A	1.4 mmol/L	1.4 mmol/L	445 U/L	<10 nmol/L	48 pmol/L	46 pmol/L	Required 5000 IU/ day of D3 for normalization
	14.7-year-old male	c.768_769insT ( <i>p.L257SfsX6</i> ) c.367 + 1 G → A	1.6 mmol/L	1.61 mmol/L	1145 U/L	<10 nmol/L	44 pmol/L	28 pmol/L	Required 10,000 IU/day of D3 for normalization
Thacher et al. [23]	12.5-year-old	Homozygous for <i>p.L99P</i>	1.48 mmol/L	0.83 mmol/L	4866 U/L	20 nmol/L	43.2 pmol/L	13.5 pmol/L	Clinical rickets not improved with 952 mg of elemental Ca daily; resolved when given 600,000 IU of D3 twice at 3- month intervals
	11-year-old	Homozygous for <i>P.L99P</i>	1.53 mmol/L	1.25 mmol/L	2391 U/L	10.25 nmol/L	40.8 pmol/L	22.9 pmol/L	Given 952 mg of elemental Ca daily as ground fish with improvement in mobility but continued mild clinical rickets
	49-year-old	Homozygous for <i>P.L99P</i>	2.4 mmol/L	1.02 mmol/L	109 U/L	12.25 nmol/L	—	6.6 pmol/L	Untreated
	5-year-old	WT/ <i>p.L99P</i>	2.4 mmol/L	0.64 mmol/L	714 U/L	41 nmol/L	432 pmol/L	11.8 pmol/L	Given 50,000 IU/ month of D2 and 952 mg of elemental Ca daily as ground fish.
	24-year-old	WT/ <i>p.K242N</i>	2.3 mmol/L	0.96 mmol/L	172 U/L	46.75 nmol/L	—	9.35 pmol/L	Untreated

Molin et al. [40]	—	Homozygous for <i>P.L99P</i>	1.98 mmol/L	1.07 mmol/L	338 U/L	<4 nmol/L	36 pmol/L	37.2 pmol/L	Treated with D3 (dose not given)
	—	Homozygous for <i>P.G42_L46delinsR</i>	1.6 mmol/L	1.4 mmol/L	762 U/L	—	—	23.8 pmol/L	Treated with alfacalcidol (1 $\alpha$ (OH)D)
Tosson et al. [41]	1.7-year-old	Unable to identify	2.15 mmol/L	0.74 mmol/L	1462 U/L	12.5 nmol/L	84 pmol/L	51.26	Treated with 1500 IU/day of and 0.25 ug/day of calcitriol (1,25(OH)D)

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*PTH*, parathyroid hormone; *WT*, wild type.



**TABLE 67.3** Basal serum 25(OH)D and peak serum 25(OH)D response to 50,000 IU of vitamin D in subjects with CYP2R1 mutations.

Publication	Genotype	Dosage	Basal 25(OH)D2 (nmol/L)	Basal 25(OH)D3 (nmol/L)	Peak 25(OH)D2 (nmol/L)	Peak 25(OH)D3 (nmol/L)	Delta 25(OH)D2 (nmol/L)	Delta 25(OH)D3 (nmol/L)
Thacher et al. [23]	<i>P.L99P</i> +/+ (n = 3)	50,000 D2	Undetectable	7.75	22.5		22.5	
		50,000 D3				32.25		24.5
	<i>P.L99P</i> -/+ (n = 5 for D3 and n = 6 for D2)	50,000 D2	Undetectable	33.5	47.5		47.5	
		50,000 D3				83.25		49.75
	<i>P.K242 N</i> -/+ (n = 1)	50,000 D2	Undetectable	39	31.0		31.0	
		50,000 D3				65.25		26.25
	WT (n = 10)	50,000 D2	Undetectable	64.8	95.5		95.5	
		50,000 D3				141.3		76.5

were homozygous for the p.L99P mutation and who were the result of a consanguineous union. A second family included an individual who was homozygous for a novel p.G42\_L46delinsR mutation. Individuals from this first family were reported to normalize their biochemical and bone defects with calcidiol treatment, whereas the individual in the second family was reported to normalize his biochemical and bone defects with alfacalcidol ( $1\alpha(\text{OH})\text{D}$ ) [40].

In addition to these reports, an additional publication describes a family with a VDDR-1B phenotype that appears to be inherited in an autosomal dominant fashion [41]. However, the authors of this publication were unable to identify any exomic or splice site mutations in the *CYP2R1* alleles.

To date, five mutations in the *CYP2R1* gene have been identified in individuals with VDDR-1B. By contrast, publicly available databases of human exome sequences indicate that there are many *CYP2R1* variants in the normal human population, including nonsynonymous nucleotide substitutions that lead to replacement of amino acids in the *CYP2R1* protein. In sum, the combined publicly available databases that include the exomic sequence for *CYP2R1* for more than 10,000 individuals (the National Heart, Lung, and Blood Institute (NHLBI) exome variant project (<http://evs.gs.washington.edu>), the ENSEMBLE sequencing project ([http://useast.ensembl.org/Homo\\_sapiens/Info/Index](http://useast.ensembl.org/Homo_sapiens/Info/Index)), and the 1000 genomes project (<http://www.1000genomes.org/>)) describe more than 200 different nonsynonymous variants (Table 67.4 includes nonsynonymous variants occurring more than 1/10,000 or clinically described nonsynonymous variants). The five mutations reported in the publications above (noted by a + in column 3, Table 67.4) represent a small proportion of the known genetic variability of *CYP2R1* (13% of the reported nonsynonymous variation); however, none of the reported nonsynonymous variants occurs in even 1% of the normal population overall, and the totality of the variants described in Table 67.4 occurs in 0.9% of all alleles or less than 2% of the population. We examined activity of the most common variants [7], and we have reported the Polyphen2 [42] and SIFT [43] predictions for each of these variants (columns 6 and 8 of Table 67.4). Of the variants that we found to alter or predict to significantly alter *CYP2R1* activity by both Polyphen2 and SIFT only p.L99P occurs as frequently as 1 in 1000 sequenced alleles. Interestingly, this mutant has a prevalence that varies significantly across populations. This mutant allele is most prevalent in areas with high UV light exposure (with a prevalence in 1000 genomes data approaching 10% in some populations indigenous to sub-Saharan Africa). We found that

this pattern was echoed in less functional variants of the vitamin D–binding protein [43]. The low rate of nonfunctional alleles overall suggests that there is significant evolutionary pressure to maintain normal *CYP2R1* activity in areas of low UV exposure. However, the increased rate of nonfunctional alleles for *CYP2R1* as well as the vitamin D–binding protein in areas of high-UV exposure suggests that these mutants may protect against vitamin D excess in this context.

The clinical phenotype of VDDR1B (summarized in Table 67.2) reveals several interesting features. First, the severity of vitamin D deficiency and clinical disease in subjects with *CYP2R1* mutations appears to lessen with age. The metabolic improvement with age may reflect the emergence of vitamin D-independent mechanism(s) for intestinal absorption of calcium that have been associated with postpubertal concentrations of reproductive hormones [44]. Alternatively, there may be a greater role for alternative 25-hydroxylases with age. And second, several options for management of VDDR-1B are available. The most common approach is to treat VDDR-1B patients with pharmacologic doses of ergocalciferol or cholecalciferol and/or calcium. An alternative approach is to bypass the defect in 25-hydroxylation by using calcitriol or recently available calcidiol. Remarkably, Molin et al. successfully treated one of their patients with alfacalcidol ( $1\alpha(\text{OH})\text{D}$ ) [40]. This approach may be effective specifically in the context of the mutation they describe (homozygous for p.G42\_L46delinsR). Another possibility is that  $1\alpha(\text{OH})\text{D}$  is a better substrate than ergocalciferol or cholecalciferol for alternate 25-hydroxylases; *CYP3A4*, for instance, has increased 25-hydroxylase activity for  $1\alpha(\text{OH})\text{D}$  over the calciferols [15].

#### 4. CYP3A4 and vitamin D–dependent rickets

Hereditary vitamin D–dependent rickets are disorders in which rickets arises from genetic defects impairing vitamin D activation or responsiveness to activated vitamin D. Until recently, these disorders were all due to loss-of-function mutations; VDDR-1B and mutations in *CYP2R1* were paradigmatic. By contrast to previously described vitamin D–dependent rickets, we recently identified a novel form of vitamin D–dependent rickets (VDDR-3; OMIM #619073) that occurs due to a gain of function mutation in *CYP3A4* [5]. Clinically, VDDR-3, similar to VDDR-1A and VDDR-1B, is characterized by early-onset rickets, reduced serum levels of the vitamin D metabolites 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, and deficient responsiveness to the parent molecule. However, differing from the clinical picture in VDDR-1A and VDDR-1B, and more

**TABLE 67.4** Predicted variant effects for common variants or for clinically described variants.

Variant	Variant ID (rs number)	Clinical phenotype described	Global minor allele frequency	Variant type	Sift class	SIFT score	Polyphen class	PolyPhen score
p.S156C	rs140947977	—	0.001	Missense variant	Deleterious	0	Possibly damaging	0.501
p.L99P	rs61495246	+	0.001	Missense variant	Deleterious	0	Probably damaging	1
p.G10V	rs374132162	—	0.001	Missense variant	Tolerated	0.56	Unknown	0
p.G10A	rs374132162	—	0.001	Missense variant	Tolerated	1	Unknown	0
p.W2R	rs199997444	—	0.001	Missense variant	Deleterious—low confidence	0	Unknown	0
p.T383A	rs112229807	—	0.0004	Missense variant	Tolerated	0.31	Possibly damaging	0.731
p.K297R	rs527423165	—	0.0004	Missense variant	Tolerated	0.16	Benign	0.003
p.M37T	rs567063734	—	0.0004	Missense variant	Tolerated	0.09	Benign	0
p.R6S	rs575551334	—	0.0004	Missense variant	Tolerated— low confidence	0.35	Unknown	0
p.G444R	rs560610153	—	0.0002	Missense variant ~ splice region variant	Deleterious	0	Probably damaging	1
p.R424Q	rs199962113	—	0.0002	Missense variant	Deleterious	0.01	Probably damaging	0.947
p.R424	rs199883994	—	0.0002	Stop gained	—	—	—	—
p.V375L	rs529613253	—	0.0002	Missense variant	Tolerated	0.21	Benign	0.441
p.C372S	rs199858102	—	0.0002	Missense variant	Tolerated	0.28	Possibly damaging	0.897
p.P359L	rs566519289	—	0.0002	Missense variant	Deleterious	0	Probably damaging	1
p.Q335R	rs139987598	—	0.0002	Missense variant	Tolerated	0.36	Benign	0.018
p.M284T	rs200183599	—	0.0002	Missense variant	Deleterious	0.02	Benign	0.107
p.D206G	rs535487358	—	0.0002	Missense variant	Tolerated	0.1	Benign	0.023
p.R145	rs576642411	—	0.0002	Stop gained	—	—	—	—
p.A140 T	rs181898397	—	0.0002	Missense variant	Tolerated	0.41	Possibly damaging	0.816
c.DNA367 + 1, G → A	rs202011621	+	0.0002	Splice donor variant	—	—	—	—
p.V100A	rs201553303	—	0.0002	Missense variant	Tolerated	0.05	—	0.692

c.-120-1G > TA	rs192940523	—	0.0002	Splice acceptor variant	—	—	Possibly damaging	—
p.S59P	rs555180485	—	0.0002	Missense variant	Tolerated	0.27	Benign	0
p.P36L	rs202122669	—	0.0002	Missense variant	Deleterious	0	Possibly damaging	0.455
p.A37V	rs553178387	—	0.0002	Missense variant	Tolerated	0.2	Unknown	0
p.A7V	rs557603109	—	0.0002	Missense variant	Tolerated—low confidence	0.95	Unknown	0
p.K242N	—	+	0.0002	Missense variant	Damaging	0.03	Possibly damaging	0.49
p.G42_L46delinsR	—	+	<0.0001	Indel	—	—	—	—
c.768insT (p.L257SfsX6)	—	+	<0.0001	Insertion	—	—	—	—



similar to VDDR-2, these patients were also seemingly resistant to activated forms of vitamin D. The two index subjects require very high doses of vitamin D supplementation or calcium supplementation to treat their clinical rickets and show decreased responses to vitamin D supplementation.

CYP3A4 oxidizes 25-hydroxyvitamin D principally via hydroxylation at the 4-beta position and inactivates 1,25-dihydroxyvitamin D via hydroxylation at the 23-R position [45,46]. Although the relevance of CYP3A4 to vitamin D homeostasis is limited under physiological conditions, previous studies indicate that under special circumstances, CYP3A4 can have important effects on vitamin D metabolism. First, induction of CYP3A4 expression by anticonvulsants and many other drugs is associated with clinically significant vitamin D deficiency via accelerated inactivation of vitamin D metabolites [45,46]. Second, targeted induction of CYP3A4 by the antibiotic rifampin has been shown to provide an alternative inactivation pathway that can normalize elevated levels of vitamin D metabolites in patients with loss-of-function mutations in *CYP24A1* [47]. Finally, common polymorphisms in CYP3A4 are associated with decreased bone density, although the mechanism remains uncertain [48].

The initial description of what has come to be known as VDDR-3 describes two unrelated female subjects from nonconsanguineous families. Both subjects initially presented to medical attention prior to the age of 2 years with clinical and radiological features of rickets including bowed legs, delayed walking, poor growth, reduced serum calcium and phosphorus, and elevated serum alkaline phosphatase and parathyroid hormone. Whole-exome sequencing followed by targeted Sanger sequencing revealed that the genetic basis for this unusual form of vitamin D resistance was identified as heterozygous gain-of-function missense mutations (p.I301T) in *CYP3A4* [5]. Functional studies revealed that recombinant CYP3A4 enzyme carrying the p.I301T mutation had tenfold increased activity to inactivate 1,25(OH)D compared with wild-type recombinant CYP3A4 and twofold increased activity compared with wild-type recombinant CYP24A1. Additionally, the p.I301T mutation decreases CYP3A4 activity for the research assay substrate luciferin IPA by nearly 50%. Expression of CYP3A4 can be induced by a wide variety of compounds that bind to the steroid and xenobiotic nuclear receptor. There is evidence of a feed-forward loop whereby bile acids, which are metabolized by CYP3A4, induce CYP3A4 [5]. Thus, the decrease in non-vitamin D catalytic activity we observe in the p.I301T mutant may serve to induce the expression of this mutant CYP3A4 and further increase 1,25-dihydroxyvitamin D<sub>3</sub> degradative activity.

## 5. In vitro and in silico analyses of CYP2R1 function

Two in vitro methods have been used to characterize CYP2R1 activity; conversion of radiolabeled parent vitamin D (ergocalciferol or cholecalciferol) to 25(OH)D in lysed or cell-free extracts or cell culture-based assays in which activity of recombinant CYP2R1 proteins is determined using a two-hybrid luciferase reporter system. The former method uses thin-layer chromatography to separate parent vitamin D from 25(OH)D, and it is an adaptation of an ex vivo method that has been in use since at least the 1960s for measurement of hepatic 25-hydroxylase activity [49]. With highly sensitive mass spectroscopy and chromatographic methods that can distinguish 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>, it is now theoretically possible to perform this cell-free assay without the use of radioactivity. Such an assay has not yet been reported in the literature. However, the second approach employs a luciferase reporter in which expression of recombinant firefly luciferase is dependent on activation of promoter sequences that contain DNA response elements that bind either the VDR or galactose 4 (GAL4)—VDR hybrid proteins consisting of a fusion between the GAL4 DNA-binding domain and the human VDR ligand-binding domain [18]. In its current iteration, this assay has been modified to enhance sensitivity by using 1- $\alpha$ (OH)-cholecalciferol as the substrate instead of cholecalciferol and a destabilized firefly luciferase (Fig. 67.1) [3,23]. Cells transfected with CYP2R1 cDNA (sequence A in Fig. 67.1) generate 1,25(OH)2D<sub>3</sub> when incubated with 1 $\alpha$ (OH)cholecalciferol; the 1,25(OH)2D<sub>3</sub> binds to the VDR ligand-binding domain in the hybrid VDR-Gal4 protein enabling the GAL4 domain to interact with copies of the yeast Gal4 upstream activator sequence in the reporter plasmid, thereby activating transcription of destabilized firefly luciferase.

The functional characteristics of only two (p.L99P and p.K242 N) of the five CYP2R1 mutants (p.L99P, p.K242N, p.G42\_L46delinsR, c.DNA367 + 1, G  $\rightarrow$  A, c.768insT (p.L257SfsX6)) that have been identified in patients with VDDR-1B have been reported [3,23,38] (Fig. 67.2). The remaining three mutants (p.G42\_L46delinsR, c.DNA367 + 1, G  $\rightarrow$  A, c.768insT (p.L257SfsX6)) are predicted to have reduced activity because of the loss or replacement of amino acids in important functional domains that have been identified by in silico analysis of the crystal structure of the CYP2R1 protein [24].

The splice site mutation (c.DNA367 + 1) is predicted to result in replacement of more than one-third of the functional CYP2R1 protein, including residues critical for the vitamin D-binding pocket (Leu125, Asn126, Phe214, Asn217, Ala221, Ala250, Val253, Tyr254, Phe302, Glu306, Ala310, Thr314, Val375, Ile379,

Met487, and Thr488) as well as residues necessary for heme coordination (Trp133, Arg137, Arg446, His381, and Ser442). Similarly, the insertion mutant leading to a premature stop codon (p.L257SfsX6) is predicted to result in omission of nearly half of the amino acids in CYP2R1 including many of the same critical amino acids for the vitamin D-binding site and heme coordination. The effect of the short insertion mutant (p.G42\_L46delinsR) is less obvious as these amino acids are all in the A' loop, which has unclear function. In the crystal structure (Fig. 67.2B—the residues for each of the three discrete mutants, p.G42\_L46delinsR, p.L99P, and p.K242N, are identified), the sequence this loop continues from is cut off to facilitate crystallization; the transmembrane portion of the protein, residues 1–31, was replaced in the crystalized protein with the amino acid sequence MAKKT. The length of the replacement in the mutant is fairly long, and it may be necessary to allow movement on the microsomal membrane or interaction with membrane components.

The p.L99P mutation is predicted by Polyphen2 [42] and SIFT [43] to result in a misfolded protein; specifically, structure modeling found that replacement of leucine99 by proline, a helix breaker, disrupts the helix that this leucine is involved in, likely resulting in an increased degradation rate. However, when this mutant protein is overexpressed in cell culture, there is no evidence of decreased protein abundance relative to the wild-type protein [23]. In addition to the structural effect that is expected by the replacement of leucine with proline in any protein, L99 is in close proximity [24] to several residues that coordinate the heme cofactor (Fig. 67.2B) and appears to form a critical hydrogen bond with Arg445 that may be necessary for appropriate positioning or electron availability for heme coordination.

Replacement of lysine242 by asparagine is predicted by SIFT [43] to be damaging and by Polyphen2 [42] to be possibly damaging to protein structure. In addition, and similar to p.L99P that has both predicted effects on protein structure generally and predicted effects on CYP2R1 function specifically, this change is predicted to destabilize positioning of Phe240 with consequent decreased interaction of CYP2R1 with its substrate.

## 6. Associations between CYP2R1 variants and disease

There is extensive genetic variation in *CYP2R1*, and many different *CYP2R1* alleles exist within the normal population, including common single nucleotide polymorphisms (SNPs) in noncoding and coding regions that do not alter the sequence of CYP2R1 as well as rare nonsynonymous SNPs in exons that result in amino

acid replacements. GWAS have consistently linked serum concentrations of 25(OH)D to SNPs in or near the *CYP2R1* locus across ethnicities [46,50–53]. Because low 25(OH)D concentrations are implicated in a number of disease states, several groups have examined whether the SNPs associated with 25(OH)D in GWAS are also associated with specific disease states; these analyses demonstrated significant associations between *CYP2R1* SNPs associated with low serum 25(OH)D and multiple sclerosis [54], type 1 diabetes [55,56], obesity [57], asthma [58,59], and elevated serum IgE [60] (Table 67.5). However, these studies have several important limitations. First, these are association studies and thus, cannot demonstrate the direction of causality. And secondly, these studies do not correct for genetic background as a confounder. Hence, it is not possible to conclude that low serum levels of 25(OH)D represent a modifiable cause of these common diseases. Specifically, to conclude that a reduced serum level of 25(OH)D represents a risk factor that actually causes these diseases, it will be necessary to demonstrate that increasing serum levels of 25(OH)D will result in a beneficial effect. The traditional approach to prove disease causation by a deficiency is through a randomized placebo-controlled interventional trial in which the deficiency is corrected. An alternative, and more expedient approach, is to use Mendelian randomization, a methodology that utilizes variation in genes of known function to examine the causal effect of a modifiable exposure on a disease or condition. The design was first described by Katan [61] as a way to understand the relationship between low cholesterol and cancer and more fully characterized by Gray and Wheatley [62] as an alternative to intervention trials for obtaining unbiased estimates of the effects of a putative causal variable. The design includes a powerful control for elimination of reverse causation and confounding effects that can reduce the reliability of conventional epidemiological studies. When performed properly, Mendelian randomization examines both the direction of causality in described associations and the strength of that association. This technique can use genetic variation to predict the outcome for a modifiable feature in subjects who have not actually had the feature measured (e.g., serum concentration of 25(OH)D) and hence can be applied to populations for which specific outcomes are known (e.g., multiple sclerosis, asthma, hypertension, etc.). Mendelian randomization for 25(OH)D uses GWAS-derived variants in *CYP2R1* as well as *GC* (encoding DBP) and *DHCR7* (encoding 7-dehydrocholesterol reductase) to generate a 25(OH)D propensity score. This score has been found to account for ~ 10%–15% of the total variation in serum levels of 25(OH)D (i.e., approximately 25 nmol/L) and is an index of the genetic predisposition toward low serum 25(OH)D. In Mendelian randomization analyses, SNPs

**TABLE 67.5** Associations between *CYP2R1* single nucleotide polymorphisms and serum levels of 25(OH)D or specific clinical disorders.

Gene or SNP	Location	Phenotype
	(Relative to <i>CYP2R1</i> )—position on chr 11	
CYP2R1	(Gene itself)—14913751–14899556	—
rs10832313	(5' promoter)—14922363	Obesity [57]
rs10766197	(5' promoter)—14921880	Serum level of 25(OH)D [69], asthma [58]
rs1562902	(5' promoter)—14,918216	Serum level of 25(OH)D [52,53,60,69], asthma [59], serum IgE [60]
rs10741657	(5' promoter)—14914653	Serum level of 25(OH)D [56], astrocytic tumors [70], type 1 diabetes [55,56], multiple sclerosis [54]
rs12794714	(5' UTR)—14913575	Serum level of 25(OH)D [52,69]
rs1993116	(5' UTR)—14910484	Serum level of 25(OH)D [50]
rs11023374	(Intron 2)—14903886	Asthma [58]
rs2060793	(3')—14866810	Serum level of 25(OH)D [50]

SNPs, single-nucleotide polymorphisms.

that are located in the *CYP2R1* promoter, via their effect on serum levels of 25(OH)D, have confirmed that reduced serum 25(OH)D levels are likely causally associated with several adverse outcomes, including multiple sclerosis, hypertension, and overall mortality [63–65]. By contrast, Mendelian randomization with SNPs within the *CYP2R1* promoter have demonstrated reverse causality for low serum levels of 25(OH)D and pediatric asthma, suggesting instead that asthma causes low circulating concentrations of 25(OH)D [66].

An important and as yet unanswered question is whether the *CYP2R1* polymorphisms that have been associated by GWAS with serum levels of 25(OH)D actually alter expression of *CYP2R1* or are in linkage disequilibrium with the true functional polymorphisms. One approach to address this question is to determine whether the SNPs are located in sequences with DNase hypersensitivity, which are generally thought to be regulatory domains, where transcription factors bind to control gene expression. Although DNA hypersensitivity sites within the human genome have been mapped by the ENCODE project, these sites have been identified using DNA from circulating blood cells and cancer cell lines, and not from cells and tissues that normally express *CYP2R1*, such as the liver and testis, which may have different DNase hypersensitivity sites. With these limitations in mind, analysis of the available ENCODE data shows that the commonly used *CYP2R1*

polymorphisms do *not* reside in DNase hypersensitivity sites and hence are likely to be nonfunctional. Rather, these SNPs may represent “tag SNPs” that are in linkage disequilibrium with the actual DNA sequences that mediate the measured effect. If the identified SNPs are tag SNPs rather than functional SNPs, then it is likely that the associations between *CYP2R1* and circulating levels of 25(OH)D are actually more significant than what has actually been measured.

## 7. Regulation of *CYP2R1* in health and disease

Vitamin D concentrations are decreased in pathologies as diverse as asthma, obesity, diabetes, and aging. These decreases have long been thought to reflect that low vitamin D predisposes to each of these pathologies. Our work using mendelian randomization to examine direction and degree of causality in the relationship between asthma and low serum vitamin D (25(OH)D) found that low serum 25(OH)D is likely an effect of asthma rather than a cause [66]. This work raises the possibility that low 25(OH)D concentrations in other diseases are effects of the disease rather than a cause.

One mechanism that might link disease to low 25(OH)D concentrations would be decreased *CYP2R1* activity. This is a controversial possibility, as *CYP2R1*

had previously been thought to be a constitutive enzyme. Our group used mouse models of aging and obesity to examine the possibility that aging and obesity decreased *CYP2R1* abundance and activity, leading to lower 25(OH)D concentrations. We found the both aging and obesity decreased liver *CYP2R1* mRNA and 25-hydroxylase activity, leading to decreased serum 25(OH)D [67,68]. Thus, we demonstrated that regulation of *CYP2R1* in the context of disease may mediate the association between some disease states and low serum 25(OH)D concentrations.

## 8. Conclusion

Recent genetic and biochemical studies of *CYP2R1* have revealed this protein to be far more important for vitamin D homeostasis than previously imagined. Heterozygous loss-of-function mutations in the *CYP2R1* gene account for vitamin D deficiency and decreased responsiveness of 25(OH)D to vitamin D supplementation, and when homozygous, cause VDDR-1B. Therefore, unlike most genes that encode important enzymes, *CYP2R1* exhibits a gene-dose effect such that there is a haploinsufficient phenotype. Perhaps even more important than the handful of *CYP2R1* mutations that have been described, ~150 nonsynonymous variants have been reported in publicly available databases, and although these variants are uncommon in the normal populations studied, there is reason to suspect that many alter function of the *CYP2R1* protein. Finally, and perhaps of greatest clinical impact, several *CYP2R1* variants have been identified by GWAS that are associated with low-circulating 25(OH)D, and which collectively are responsible for 20%–30% of the genetic contribution to serum levels of 25(OH)D. Moreover, these variants have been used to confirm causal relationships between reduced circulating levels of 25(OH)D and several common diseases, such as obesity, asthma, and multiple sclerosis, as well as overall mortality. Understanding how these variants affect *CYP2R1* activity and lead to disease may suggest innovative approaches to improving health. Although genetic variation has been a larger focus of research, recently, we have reported three lines of evidence suggesting that in specific contexts (i.e., asthma, aging, and obesity), transcriptional regulation of *CYP2R1* has clinically significant effects on its activity and serum 25(OH)D.

By contrast to *CYP2R1*, in normal conditions, *CYP3A4* does not play a prominent role in vitamin D homeostasis. However, a rare gain-of-function mutation in *CYP3A4* has recently been found to cause VDDR-3. Additionally, induction of *CYP3A4* incidentally by anti-convulsants and many other drugs or therapeutically by rifampin increases inactivation of forms of vitamin D.

## 9. Summary points

- *CYP2R1* is the principal vitamin D 25-hydroxylase.
- Homozygosity for complete loss-of function mutations in *CYP2R1* causes VDDR-1B.
- Heterozygosity for complete loss-of function mutations in *CYP2R1* causes a vitamin D deficiency where individuals have decreased responsiveness to supplementation.
- An extremely rare gain-of-function mutation in *CYP3A4* causes VDDR-3.
- In specific contexts, inhibition or induction of transcription or activity of either *CYP2R1* and *CYP3A4* has clinically significant effects on vitamin D homeostasis (Tables 67.1–67.5).

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## Further reading

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# Hereditary 1,25-dihydroxyvitamin D resistant rickets (VDDR-2A)

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## OBJECTIVES

- To present the clinical picture of hereditary vitamin D resistant rickets (HVDRR) including the family setting and the role of consanguinity.
- Discuss the various mutations that cause HVDRR and how their location in the VDR provides information on the function of various segments of the VDR protein.
- Cover the various forms of treatment as they have evolved and expanded.
- Discuss the phenomenon of spontaneous improvement in the clinical picture around puberty and the hypothesis of how pubertal hormones may improve calcium absorption by a vitamin D-independent action.
- Examine the unusual finding of alopecia in some but not all cases of HVDRR and discuss current research to explain the mechanism and which mutations are associated with the problem.
- There is great interest today in the possible action of vitamin D to reduce the incidence and severity of many extra-skeletal diseases. Children with genetic defects that prevent vitamin D action from birth would be expected to suffer an increased incidence of these illnesses. We examine the limited number of cases being followed to determine whether they appear to have an increased risk of cancer, autoimmune disease, or other maladies including effects on reproduction.

## 1. Introduction

As discussed in many chapters in this book, vitamin D is the primary regulator of calcium homeostasis in the body and is particularly important in skeletal development and in bone mineralization. The hormonally active, form of vitamin D,  $1\alpha, 25$ -dihydroxyvitamin D [ $1,25(\text{OH})_2\text{D}$  or calcitriol], functions like other steroid hormones by binding with high affinity to its specific receptor, the vitamin D receptor (VDR). The VDR is a member of the steroid-thyroid-retinoid receptor gene superfamily of nuclear transcription factors that regulate the expression of specific target genes in response to hormone binding. Hereditary  $1,25(\text{OH})_2\text{D}$ -resistant rickets (HVDRR), also known as vitamin D-dependent rickets type 2A (VDDR-2A), is a rare genetic disease that is due to a generalized resistance to  $1,25(\text{OH})_2\text{D}_3$  action. HVDRR is caused by heterogeneous mutations in the VDR gene that cause loss of function of the receptor ultimately leading to complete target organ resistance to  $1,25(\text{OH})_2\text{D}$  action. In this chapter, we update our previous reviews on HVDRR [1–8], describe the clinical manifestations of the disease, and discuss the published genetic defects in the VDR underlying the molecular basis for HVDRR adding newly described mutations and current research.

Over the years several different names have been used to describe the condition caused by  $1,25(\text{OH})_2\text{D}$  resistance (when written without the  $\text{D}_2$  or  $\text{D}_3$  subscript we mean either/or both forms of vitamin D). In addition to the designation HVDRR, the disease has been referred to as vitamin D-dependent rickets type II (VDDR-II),



**TABLE 68.1** Comparison of VDDR-1's, VDDR-3 and HVDRR (VDDR-2A).

Mechanism	Synthesis defective		Receptor defective	Increased degradation
Descriptive Designation	1 $\alpha$ -hydroxylase Deficiency	25-hydroxylase Deficiency	HVDRR	Accelerated Catabolism
OMIM designation	VDDR-1A	VDDR-1B	VDDR-2A	VDDR-3
Gene mutated	CYP27B1	CYP2R1	VDR	CYP3A4
Recessive mutation	Yes	Yes	Yes	Dominant <sup>c</sup>
Appears early age	Yes	Yes	Yes	Yes
Rickets	Yes	Yes	Yes	Yes
Elevated Alk Phos	Yes	Yes	Yes	Yes
Hypocalcemia	Yes	Yes	Yes	Yes
Alopecia	No	No	Some patients <sup>a</sup>	No
PTH	Elevated	Elevated	Elevated	Elevated
25(OH)D levels	Normal or low	Low	Normal	Low
1,25(OH) <sub>2</sub> D levels	Low	Low	Elevated	Low
Response to doses of 1,25(OH) <sub>2</sub> D	Yes	Yes	No (with exceptions) <sup>b</sup>	Requires high dose <sup>d</sup>

<sup>a</sup>Alopecia is associated with mutations that affect DNA binding, VDR-RXR heterodimerization, or that truncate the VDR.

<sup>b</sup>Mutations that reduce the affinity for 1,25(OH)<sub>2</sub>D<sub>3</sub> can sometimes be overcome by treating with elevated concentrations of various vitamin D ligands.

<sup>c</sup>The missense mutations in CYP3A4 are de novo and dominant and are not inherited from the parents.

<sup>d</sup>The dose required to maintain adequate 1,25(OH)<sub>2</sub>D<sub>3</sub> is supra-physiologic that overcomes the accelerated degradation created by the mutation in CYP3A4.

pseudovitamin D deficiency rickets type II (PDDR-II), calcitriol-resistant rickets (CRR) and hypocalcemic vitamin D-resistant rickets (HVDRR). In the Online Mendelian Inheritance in Man web site (<http://www.ncbi.nlm.nih.gov/omim/>) this disease is now referred to as Vitamin Dependent Rickets-2A (VDDR2A). We prefer to use the designation "Hereditary 1,25-dihydroxyVitamin D Resistant Rickets" (HVDRR) since this disease is now known to be caused by genetic defects in the VDR that lead to resistance to the action of 1,25(OH)<sub>2</sub>D (not dependence on) and this simplified name is the term most used in previous publications [3,5–10]. However, there is a recent trend in some publications to use the VDDR terminology instead of HVDRR, so we will use both terms in Table 68.1 and intermittently in the text but mostly use HVDRR, the name that we believe more correctly defines the mechanism of the disease.

In summary, the VDDR terminology is as follows and is described more completely in Table 68.1 and discussed by Levine [11]. VDDR-1 is caused by mutations in genes encoding the enzymes that metabolize vitamin D to 25-hydroxyvitamin D (25(OH)D) and 1,25(OH)<sub>2</sub>D: CYP2R1 and CYP27B1. VDDR-2 (HVDRR is also known as VDDR-2A), is caused by mutations in genes encoding essential signal transducing proteins: VDR and heterogeneous nuclear

ribonucleoprotein (hnRNP) VDDR-2B. VDDR-3 is due to gain-of-function mutations in a gene encoding a vitamin D degrading enzyme CYP3A4.

Historically, the notion that some cases of rickets could be due to hormone resistance was put forward in 1937 by Albright et al. [12]. They described a patient with rickets and normal serum calcium levels but low phosphate levels who responded to treatment with very high doses of vitamin D. This led the authors to suggest that the cause of the condition was due to end-organ resistance to vitamin D and thus the concept of target gland hormone resistance developed. The patient they described appears to have had what is now known as X-linked hypophosphatemic rickets (XLH), described in Chapter 64. Twenty-four years later, Prader et al. [13] reported on two patients with rickets who were hypocalcemic and hypophosphatemic. These patients also responded to high doses of vitamin D and they referred to this condition as vitamin D-dependent rickets type I (VDDR-I). The cause of rickets in these individuals was eventually discovered to be due to an inborn error in the enzyme that converts vitamin D to the hormonally active form 1,25(OH)<sub>2</sub>D. It is now well established that VDDR-1A arises from mutations in the CYP27B1 gene that encodes the enzyme 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase) that converts 25-hydroxyvitamin D

[25(OH)D] to 1,25(OH)<sub>2</sub>D [4,14,15]. This disease is described in Chapter 66.

Defects in the first step of vitamin D activation, the conversion of vitamin D to 25(OH)D, are due to mutations in the gene responsible for the vitamin D 25-hydroxylation step (*CYP2R1*). This entity has now been designated as VDDR-IB and is discussed in Chapter 67.

A second mutation in the VDDR-2 category in addition to HVDRR is a defect seen so far as we are aware in only a single patient [16]. The defect is in heterogeneous nuclear ribonucleoprotein (hnRNP) that binds to hormone response elements and thereby can cause vitamin D resistance [16,17].

A recently described form of genetic rickets is termed VDDR-3. This disease is due to a gain of function mutation in the *CYP3A4* gene leading to accelerated degradation of vitamin D metabolites [18] (and Chapter 67). Although very few patients with *CYP3A4* mutations have been identified as yet, a similar mechanism may be important in drug-induced osteomalacia since some commonly used drugs stimulate *CYP3A4* activity leading to accelerated vitamin D metabolite degradation [19]. The VDDR terminology is added to Table 68.1 to describe and compare these diseases.

Focusing on HVDRR (VDDR-2A), in 1978, the first case reports of actual HVDRR were published by Brooks et al. [20] and Marx et al. [21]. The patient in the Brooks et al. study was both hypocalcemic and hypophosphatemic and had secondary hyperparathyroidism. Many clinical findings were similar to patients with VDDR-IA except that the patient had markedly increased serum levels of 1,25(OH)<sub>2</sub>D. Brooks et al. postulated that the rickets was due to end-organ resistance to 1,25(OH)<sub>2</sub>D and they named the syndrome vitamin D dependent rickets, type II (VDDR-II). Marx et al. reported similar findings in two children and also suggested that the disease was due to end-organ resistance to 1,25(OH)<sub>2</sub>D. Since these initial studies, although the condition is rare in the general population, there have been many reports of patients with HVDRR due to target organ resistance to 1,25(OH)<sub>2</sub>D action [1–10,22–24] especially from areas of the world where consanguinity is common, often in isolated communities. In this review, we will update the clinical features and the genetic basis underlying the disease and we attempt to include all of the published mutations up to the time of writing this chapter as well as reviewing new research findings.

## 2. Clinical features

### 2.1 Clinical and biochemical findings and distinguishing features

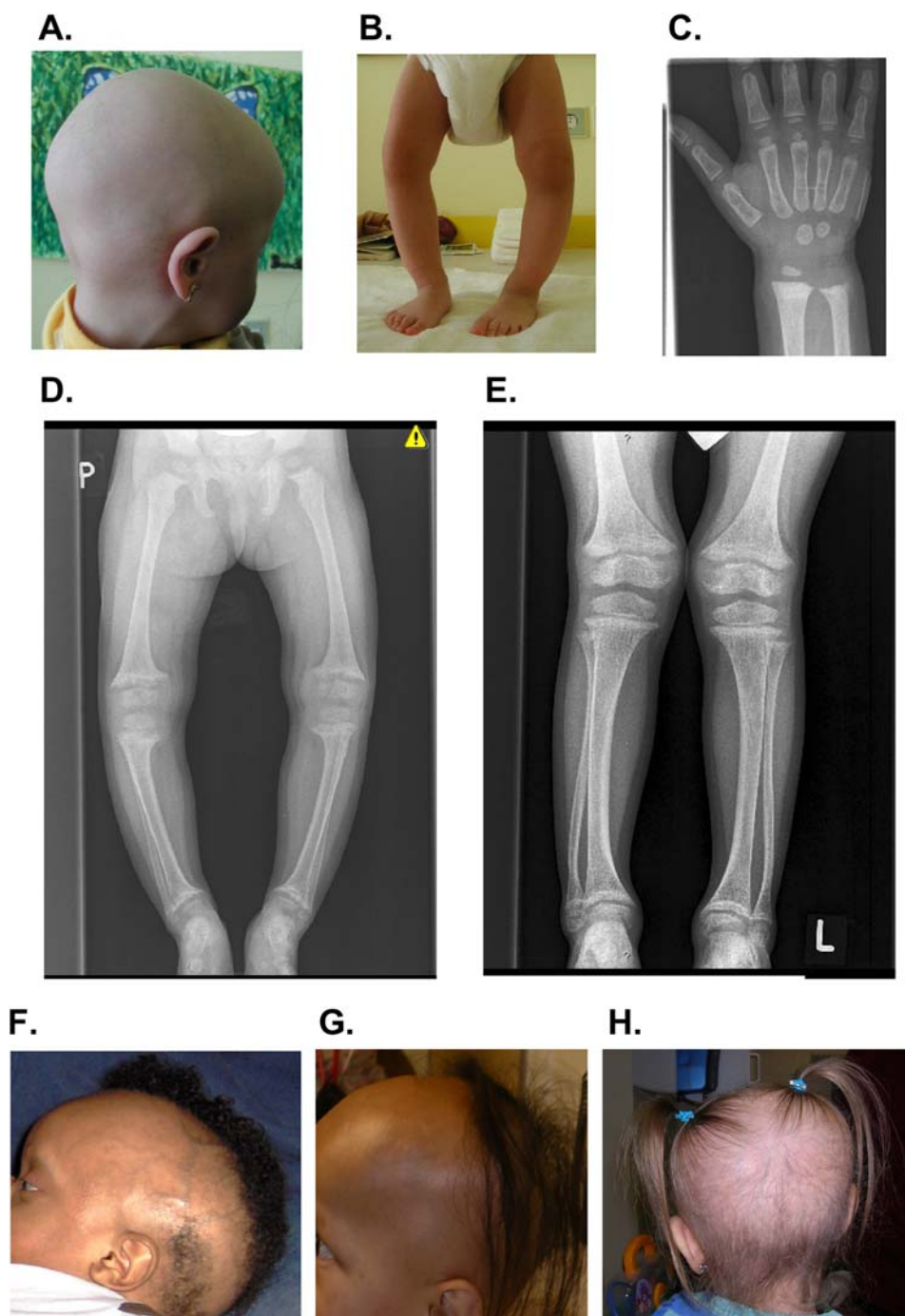
HVDRR (or VDDR-2A) is manifested by a constellation of signs and symptoms caused by generalized

resistance to 1,25(OH)<sub>2</sub>D action and by a loss of ligand-dependent and possibly ligand-independent actions of the VDR. The main features of HVDRR are severe rickets with osteomalacia, hypocalcemia, secondary hyperparathyroidism, hypophosphatemia, and elevated alkaline phosphatase. Some patients have total or partial alopecia. At birth or soon after, hair is lost and not replaced. The development of partial or complete alopecia may be the only early obvious symptom (Fig. 68.1). Alopecia, and which patients develop it, will be discussed in greater detail below in Section 8.

Calcium levels are often within normal limits soon after birth, due to adequate transfer of calcium from the mother to the fetus. Biochemically, the earliest evidence of HVDRR is often elevated serum concentration of 1,25(OH)<sub>2</sub>D. This is an inherent part of the disease due to the loss of the down-regulating effect that calcitriol has on the *CYP27B1* enzyme through VDR action, as well as the development of secondary hyperparathyroidism due to falling serum calcium levels. Hypocalcemia results in elevated parathyroid hormone (PTH) secretion and thereby increased stimulation of *CYP27B1* expression resulting in elevated production of 1,25(OH)<sub>2</sub>D [25]. During the first months of life, phosphaturia, due to secondary hyperparathyroidism, is followed by a decline in serum phosphorus. In the following months, severe rickets, with hypocalcemia and hypophosphatemia, develop rapidly. The potential role of fibroblast growth factor 23 (FGF23) or other phosphatonins involved in the phosphate abnormality, or in the elevated serum 1,25(OH)<sub>2</sub>D concentration are discussed in Chapters 19 and 64. If not treated, the growth of the child is severely attenuated and bowing of the long bones and fractures can develop. X-ray studies show classical changes in rickets (Chapter 62).

In HVDRR-affected children, signs of rickets generally appear early, usually within months of birth. The rickets is often severe and affected children suffer from bone pain, muscle weakness, and hypotonia. In the worst cases, convulsions due to the hypocalcemia have occurred. Children are usually growth retarded and they often develop severe dental caries or exhibit enamel hypoplasia of the teeth [26–30]. Some infants have died from pneumonia as a result of poor respiratory movement due to severe rickets of the chest wall [27,28,31]. In many cases, children with HVDRR have sparse body hair and some have total scalp and body alopecia including eyebrows and in some cases eyelashes (Fig. 68.1). Alopecia, and why some cases do not exhibit alopecia, will be discussed in more detail below (sub-Section 3 and Section 8).

The clinical findings in HVDRR (VDDR-2A) are shown in Table 68.1 and compared to three other genetic causes of early onset rickets due to enzymatic defects in the synthetic pathway of 1,25(OH)<sub>2</sub>D, (VDDR-1), or in the case of VDDR-3, accelerated degradation of



**FIGURE 68.1** Alopecia and rickets in children with HVDRR. (A) Patient F70 with total alopecia; (B) the child exhibited bowed legs; (C) X-ray of wrist; (D) X-ray of bowed legs; (E) X-ray of legs after 4 year of calcium and calcitriol therapy. HVDRR children with partial alopecia. (F) Patient F69; (G) patient F78; (H) patient F79. Panels (A–E) Reproduced from [22] with permission from the American Society for Bone and Mineral Research. Panel (F) reproduced with permission from [23]. Panels (G and H) reproduced with permission from [24].

25(OH)D and 1,25(OH)<sub>2</sub>D. In comparison with HVDRR (VDDR-2A), Table 68.1 shows the usual finding in the defects that block the 1 $\alpha$ -hydroxylase or the 25-hydroxylase, enzymes (VDDR-1) that would normally activate vitamin D to 1,25(OH)<sub>2</sub>D. Table 68.1 also includes VDDR-3 due to increased degradation of the

active vitamin D metabolites. The common clinical abnormalities in all of these diseases include low serum concentrations of calcium and phosphate and elevated serum alkaline phosphatase activity (Alk Phos). The mechanism is an inadequate synthesis of 1,25(OH)<sub>2</sub>D due to synthetic enzyme mutations (VDDR-1), or

increased degradation of the active metabolites (VDDR-3). The inability of even the elevated  $1,25(\text{OH})_2\text{D}$  to activate target genes is the mechanism in the case of VDDR-2A (HVDRR) where *VDR* gene mutations disables the VDR. All four conditions cause secondary hyperparathyroidism because of hypocalcemia. In HVDRR, the inability of the elevated  $1,25(\text{OH})_2\text{D}$  levels to activate the VDR to suppress PTH, contributes to the hyperparathyroidism. The elevated PTH level then exacerbates hypophosphatemia. Many of these clinical and biochemical findings are common to patients with the various forms of VDDR as shown in Table 68.1 and are described in detail in Chapters 66 and 67. The most obvious differences are that VDDR-2 patients have elevated  $1,25(\text{OH})_2\text{D}_3$ , may have alopecia, and most often are unresponsive to treatment with vitamin D.

The third and more recently recognized genetic cause of rickets designated VDDR-3 is discussed in Chapter 67. In this disease, children with early onset rickets, show reduced serum levels of the vitamin D metabolites  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$ , and deficient responsiveness to vitamin D or its hydroxylated forms [18]. The patients do not have any mutations in the enzymes that cause VDDR-1 rickets or in the VDR that causes HVDRR (VDDR-2A). However, using whole exome sequencing analysis, missense mutations were identified in *CYP3A4*, an enzyme that inactivates vitamin D metabolites [18]. The mutation occurs in the substrate recognition site resulting in a gain of function that substantially increased the ability of the mutant enzyme to specifically inactivate activated vitamin D metabolites. The mutant *CYP3A4* even exhibits twofold greater activity than *CYP24A1*, the major physiological inactivator of vitamin D metabolites. These findings provide insight into vitamin D metabolism and demonstrate that accelerated inactivation of vitamin D metabolites represents a mechanism for vitamin D deficiency [18].

In HVDRR patients the serum  $25(\text{OH})\text{D}$  values may be normal and the  $1,25(\text{OH})_2\text{D}$  levels are elevated, often substantially [9,32]. This singular feature distinguishes HVDRR from  $1\alpha$ -hydroxylase deficiency (VDDR-1A),  $25$ -hydroxylase deficiency (VDRR-1B), and VDDR-3, where the serum  $1,25(\text{OH})_2\text{D}$  values are depressed or even may be undetectable due to loss of *CYP27B1* or *CYP2R1* activity or increased *CYP3A4* activity. Another crucial difference is that patients with VDDR-1A deficiency can be successfully treated with physiologic doses of calcitriol, or  $25(\text{OH})\text{D}$  in the case of VDRR-1B, that bypass the enzymatic defects and restore the circulating  $1,25(\text{OH})_2\text{D}$  levels to normal. In the case of VDDR-3, higher physiologic doses are necessary to treat the condition. In contrast, patients with HVDRR exhibit elevated endogenous levels of  $1,25(\text{OH})_2\text{D}$  and do not respond to additional supra-physiologic doses of calcitriol and most patients are resistant to even extremely

supra-physiologic doses of all forms of vitamin D therapy. However, some patients, mostly with ligand-binding domain (LBD) mutations, may respond to high doses of calcitriol, as described below in Section 6 on Therapy.

HVDRR (VDDR-2A) is inherited as an autosomal recessive disease. The recessive nature of the disease is evident from the patient's parents and most siblings, who are heterozygous for the genetic trait but most often show no symptoms of the disease and have normal bone development. In most cases, consanguinity in the family lineage can be found and intermarriage is highly associated with the disease. Males and females are equally affected and often a family has several affected children [33]. In some unusual cases, there is no history of consanguinity and a different mutation has been inherited from each parent (compound heterozygous mutations). While a majority of cases are due to autosomal recessive transmission, there are now two reports describing autosomal dominant transmission where the mutant VDR exerts dominant negative activity over the wild-type VDR [34,125]. The genetics of VDDR-3 is different. In this condition, the mutations are de novo dominant mutations in the *CYP3A4* gene in affected children from families where the parents and other children do not have mutations in *CYP3A4* or the synthetic enzymes or the *VDR* gene [18].

## 2.2 Pathophysiology

In HVDRR (VDDR-2A), the intestine, and all other vitamin D target organ's including bone, parathyroid glands, and kidneys, are resistant to  $1,25(\text{OH})_2\text{D}$  action. Without vitamin D action, the intestine becomes less efficient in promoting calcium and phosphate absorption into circulation. It is now well established that the biological actions of  $1,25(\text{OH})_2\text{D}$  are mediated by binding to the VDR, a nuclear transcription factor that regulates gene expression in  $1,25(\text{OH})_2\text{D}$ -responsive cells (see several chapters in Section 2 of this volume for details).

Historically, the primary biological processes attributed to vitamin D were the maintenance of calcium and bone homeostasis.  $1,25(\text{OH})_2\text{D}$  is essential for promoting the transport of calcium and phosphate across the small intestine and into the circulation (Chapter 18). Adequate delivery of calcium and phosphate to the bone is essential for the normal mineralization of bone (Chapter 21). Approximately half of the total calcium absorption by the intestine is attributed to  $1,25(\text{OH})_2\text{D}$  action while passive absorption and nonvitamin D-mediated actions account for the remaining half [35,36]. Hypophosphatemia results from the elevated PTH down-regulating the Na/P co-transporter and/or by the loss of a functional VDR in the kidney as well



as decreased intestinal absorption. The absence of an effect of  $1,25(\text{OH})_2\text{D}$  to normally regulate FGF23 or other phosphatonins to normalize phosphaturia and  $1\alpha$ -hydroxylase activity may also play a role (Chapters 19 and 64). The calcium and phosphate deficiencies compromise normal bone mineralization leading to rickets in children and osteomalacia in adults.

There is now a large body of evidence documenting multiple actions of vitamin D in many if not all extra-skeletal organs that are discussed in many chapters of this book. Current evidence for the clinical relevance of some of these actions in patients with HVDRR will be discussed below in [Section 9](#).

## 2.3 Alopecia

Alopecia (sometimes called atrichia) is a clinical feature that is found in many but not all patients with HVDRR. This feature is unique to HVDRR and is not seen in VDDR-1 or VDDR-3 or nutritional vitamin D deficiency. Some affected patients have sparse body hair and some exhibit total scalp and body alopecia in various patterns ([Fig. 68.1](#)) [26,37–39]. Children with extreme alopecia often lack eyebrows and in some cases eyelashes. Hair loss may be evident at birth or occurs during the first few months of life as some hair the child was born with falls out and is not replaced. An analysis of HVDRR patients shows that there is some correlation between the severity of rickets and resistance to vitamin D and the presence of alopecia [39]. Patients with alopecia are generally more resistant to therapy with vitamin D or its more active analogs than those without alopecia. In families with a prior history of the disease, the absence of scalp hair in newborns provides initial diagnostic evidence for HVDRR. The mechanism causing alopecia is being unraveled ([Section 8](#) on Alopecia below and [Chapter 25](#)). However, since VDRs are present in the hair follicle [40,41] and certain mutations in the VDR cause alopecia, but vitamin D deficiency does not, the data indicate a link specifically to the unliganded VDR [42]. A skin biopsy from one patient with HVDRR and alopecia showed apparently normal follicles with no hair [38] while other patients exhibited an absence of normal hair follicles and the presence of follicular remnants and cysts [43,44]. This latter observation led the authors to conclude that the alopecia in HVDRR patients was a phenocopy of generalized atrichia with papular lesions (APL) a genetic disorder caused by mutations in the *hairless* (*hr*) gene [45,46]. Other studies led to further speculation that the VDR and HR converge to regulate similar pathways in the hair cycle [43,44]. A ligand-independent action of the VDR has been proposed to be critical during hair follicle development and therefore mutations in the VDR that

disrupt these ligand-independent actions are suspected to cause alopecia [32,47]. Patients with VDDR-1 or VDDR-3 mutations or with other causes of vitamin D deficiency do not have alopecia, further supporting the role of a ligand-independent action of the VDR during the hair cycle. Alopecia is discussed in more detail below in [Section 8](#) and in [Chapter 25](#).

## 3. Mechanism of $1,25(\text{OH})_2\text{D}$ action relevant to HVDRR (VDDR-2A)

### 3.1 The vitamin D receptor

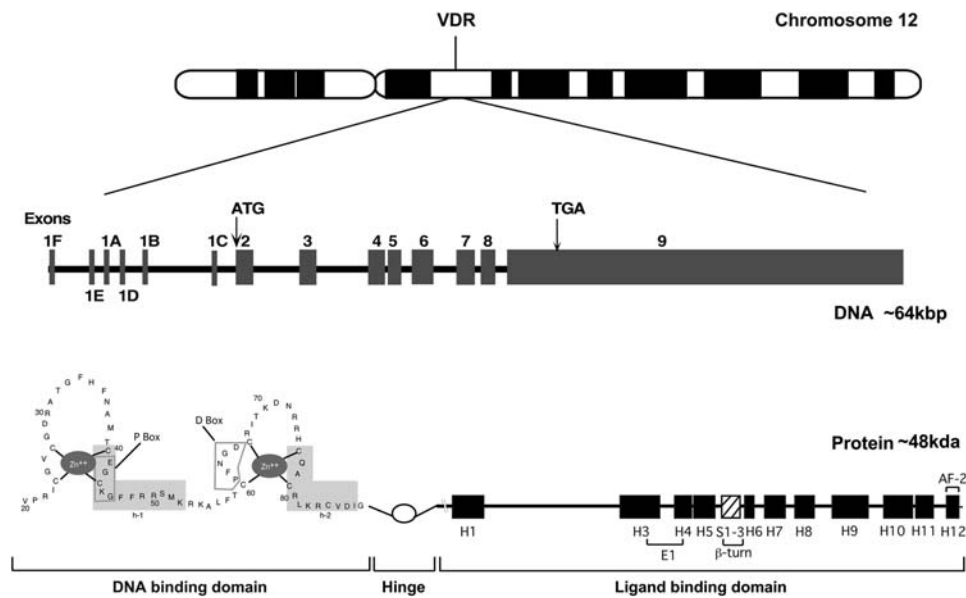
The human VDR gene is located on chromosome 12q13.11 and is composed of 14 exons spanning ~64 kbp of DNA (Chapters 10–13). The human VDR protein is composed of either 427 or 424 amino acids depending upon the presence of a T to C polymorphism (ATG to ACG) in the 427 translational start site that can be identified by an *FokI* restriction site [48,49]. Longer isoforms of 450 and 477 amino acids are also possible due to the differential splicing of exon 1D [50]. The overall structure of the VDR protein is very similar to the other members of the steroid-thyroid-retinoid receptor superfamily.

A highly conserved two zinc-finger DNA-binding domain (DBD) is located in the N-terminus of the VDR ([Fig. 68.2](#)). The DBD is encoded by exons 2 and 3 and is composed of two finger-like modules of 12–13 amino acids each. Four cysteine residues in each finger-like module bind one zinc atom to form the two zinc-finger configuration [51,52]. Specific regions of the DBD are critical for DNA binding, as well as providing a dimerization interface for interaction with the retinoid X receptor (RXR). A nuclear localization signal is also present in the DBD [53,54].

The VDR ligand-binding domain (LBD) is encoded by exons 4–9 [51]. The LBD is composed of 12  $\alpha$ -helices (H1–H12) and 3  $\beta$ -sheets (S1–S3) ([Fig. 68.2](#)) [55]. A hinge region (amino acids 88–122) provides the proper spacing between the DBD and LBD and is important in binding to the nuclear matrix and transcriptional activation [56–58].

### 3.2 Regulation of gene expression by $1,25(\text{OH})_2\text{D}$

The mechanism of action of vitamin D is detailed in multiple chapters in this book. Briefly, after  $1,25(\text{OH})_2\text{D}$  is synthesized in the kidney, it circulates in the blood mostly bound to the vitamin D binding protein (DBP) and perhaps other carriers with a small fraction of hormone in the free state. The free, fat-soluble hormone is believed to enter target cells through the



**FIGURE 68.2** Arrangement of the chromosomal gene and domains of the VDR. The structural organization of the human VDR gene which spans approximately 64 kilobases of DNA is shown [51]. The locations of the start (ATG) and termination (TGA) codons are indicated. The exons encoding the various domains and structural motifs in the VDR protein are shown. The VDR DNA binding domain is comprised of two zinc-finger modules each of which contains 4 invariant cysteine residues that function to coordinate a single zinc atom. Two  $\alpha$ -helices (helix A and B) shaded in the diagram are located on the carboxy-terminal side of each zinc module. Amino acid residues essential to functional interaction of these  $\alpha$ -helices with either DNA or with RXR are boxed and designated the P-box and D-box, respectively. In the ligand-binding domain, the position of the  $\alpha$ -helices (H1–H12) and  $\beta$ -turns (S1–3) are shown as shaded and hatched boxes respectively. The E1 and AF-2 regions are indicated.

lipid bilayer of the cell membrane although additional complex entry mechanisms may play a role, including endocytosis of DBP (see Chapter 7). Many target tissues also possess the enzymatic machinery to synthesize  $1,25(\text{OH})_2\text{D}$  locally within the cell from circulating  $25(\text{OH})\text{D}$  or from cholesterol precursors. Once inside the cell by either pathway,  $1,25(\text{OH})_2\text{D}_3$  binds to the VDR and initiates heterodimerization with RXR and translocation to the nucleus [59]. The ligand-bound VDR-RXR heterodimer binds with high affinity through their respective DBDs to vitamin D response elements (VDREs) located in regulatory regions of target genes [60]. In the LBD,  $1,25(\text{OH})_2\text{D}_3$  makes contact with specific amino acid residues lining the ligand-binding pocket [55] triggering the recruitment of coactivators and other transcription factors [61]. The VDR-RXR coactivator complex then interacts with the general transcription apparatus and drives the transcription of  $1,25(\text{OH})_2\text{D}$ -responsive genes that ultimately determines the cellular response to the hormone. Details of the structural interactions of the hormone with the VDR as well as some HVDRR mutant VDRs are shown and discussed in Chapter 10.

There is some evidence for ligand-independent actions of the VDR [42] which may be the mechanism for VDR mutations causing alopecia. Since alopecia is not found with vitamin D deficiency or other genetic causes of rickets, a role for the unliganded VDR appears to be

the best explanation. This is further discussed in Chapter 25 and below in Section 8 on alopecia.

Proof that the cause of HVDRR is due to defects in the VDR was initially developed through studies of HVDRR patients that showed defective  $1,25(\text{OH})_2\text{D}_3$  induction of target genes such as 24-hydroxylase [62–64]. Mutant VDRs identified in HVDRR patients were incapable of activating VDRE-promoter constructs supporting the critical role of functional VDR in transactivation as well as defining the defects causing HVDRR [65–67]. The development of an animal model of HVDRR by silencing and mutating the VDR was very helpful in understanding the pathophysiology of the HVDRR syndrome as described in Chapter 30.

## 4. Cellular basis of HVDRR (VDDR-2A)

### 4.1 Initial studies using cultured skin fibroblasts

The clinical picture of HVDRR was first recognized as an entity in 1978–79 [20,21,26,68]. Since that time more than 100 patients with HVDRR have been studied and an updated summary of these cases is shown in Table 68.2. Throughout this chapter, the HVDRR cases are denoted by a family number, e.g., F1, F2, etc., and are tabulated with references in Table 68.2. Studies to elucidate the nature of the molecular defect in HVDRR cases began soon after receptors for  $1,25(\text{OH})_2\text{D}$  were

TABLE 68.2 Compilation of HVDRR cases.

Family	Patient name/description	Ethnic origin	Parents related	Gender	Alopecia	VDR mutation	References
F1	IIB, patient 1, 1a		No	M	No		[21,69,70]
	IIC, patient 2, 1b		No	F			[21,69]
F2	Patient		No	F	No		[20]
F3	Patient 1, patient 2a, 2a		Yes	F	Yes		[26,69,71,72]
	Patient 2, patient 2b, 2b		Yes	F			[26,69–71]
F4	Patient		No	F	No	Ile314Ser	[68,73,74]
F5	Patient, patient 3, 3, kindred 3, P3		Yes			Arg80Gln	[27,70,71,75]
F6	K.N.	Japanese	Yes				[76,77]
F7	Patient		No	M	Yes		[78]
F8	Patient		?	M	?		[79]
F9	M.A., kindred 6, patient 6, 6	Arab	No	F	Yes		[71,80]
F10	Patient		No	F	No		[81]
F11	I.H., A1, patient 2, I.K., case 1	Arab	Yes	M	Yes	Tyr295stop	[37,62,64,82]
	R.K., patient 1, A2, case 2		Yes	F	Yes	Tyr295stop	[37,38,64,82]
F12	Patient 4		Yes		Yes		[71,83]
F13	Patient 5		Yes		Yes		[71,83]
F14	Patient		Yes	M	No		[84]
F15	Patient A, patient 5		Yes	M	Yes		[28]
F16	Patient B, patient 4		Yes	F	Yes		[28]
F17	B Patient		?		Yes		[37]
F18	S.H., patient 3, case 3, C1	Arab	No	M	Yes	Tyr295stop	[38,64,66,80]
	R.H., patient 4, case 4, C2		No	M	Yes	Tyr295stop	[38,64,66,80]
F19	D1	Haitian	Yes	F	Yes	Arg73Gln	[63,65]
	D2		Yes	F	Yes	Arg73Gln	[63,65]
F20	Kindred 7, patient 7, 7, P7			F	Yes	Arg 80Gln	[70,72,75]
F21	I.S., patient	Kuwait	Yes	M	No	Arg274Leu	[31,85,86]
F22	Patient	Hispanic	No	M	Yes		[76]
F23	Patient	Saudi	Yes	M	Yes	Gly46Asp	[87,88]
F24	Patient 1, N.D.	Arab	Yes	M	Yes		[89,90]

F25	Patient 1	Japanese	Yes	F	Yes		[91]
F26	Patient 2	Japanese	No	F	Yes	Arg50Gln	[49,91]
F27	Patient 3	Japanese	No	M	Yes		[91]
F28	Patient 2, M.T.	Persian-Jewish	Yes	M	Yes		[89,90]
F29	Patient, line 10	Saudi	Yes	M	Yes	Tyr295stop	[30,72,92]
F30	Line 15	Saudi				Tyr295stop	[92]
F31	G1	Arab	Yes	M	Yes	Gly33Asp	[65,93]
	G2		Yes	M	Yes	Gly33Asp	[65,93]
F32	Line 11, patient 1	Turkish	Yes	F	Yes	Gln152stop	[72,86,94]
	Line 11b, patient 2		Yes	M	Yes	Gln152stop	[92,94]
F33	Patient 1, patient 1a, patient 4a	Japanese	Yes	M	Yes	Arg50Gln	[49,95]
	Patient 2, patient 1b, patient 4b		Yes			Arg50Gln	[49,95]
F34	E1	Arab	Yes	M	Yes	Tyr295stop	[33,66]
F35	F1	Arab	Yes	F	Yes	Tyr295stop	[33]
F36	H1	Arab	No	F	Yes	Tyr295stop	[33,66]
F37	J1	Arab	No	M	Yes	Tyr295stop	[33]
F38	K1	Arab	No	M	Yes	Tyr295stop	[33]
F39	L1	Arab	Yes	F	Yes		[33]
F40	Ro-VDR, brother			M	No		[96]
	Al-VDR, sister			F	No		[96]
F41	Ab-VDR			M	Yes		[96]
F42	Patient					Exon 7–9 deletion	[97]
F43	Child					Cys190Trp	[97]
F44	Patient II, case 2	Tunisian	Yes	M	Yes	Lys45Glu	[98,99]
F45	Propositus	Japanese-Brazilian	Yes	M	Yes	His35Gln	[100]
F46	Line 14	Moroccan	Yes		Yes	Arg73stop	[92]
F47	Patient I	Mauritius	No	F	Yes	Phe47Ile	[98,101]
F48	J.K.	English	No	None			[16,17]
F49	N1	Tunisian-Jewish	Yes	M	Yes	Arg80Gln	[102]
	N2		Yes	F	Yes	Arg80Gln	[102]
F50	Patient	Greek	No	F	Yes	IVS3+5 (G > C)	[103]

Continued



TABLE 68.2 Compilation of HVDRR cases.—cont'd

Family	Patient name/description	Ethnic origin	Parents related	Gender	Alopecia	VDR mutation	References
F51	Patient	Turkish	Yes	M	No	His305Gln	[104,105]
	Sister		Yes	F	No	His305Gln	[104,105]
F52	Patient 2	French-Canadian	Yes	F	Yes	Arg391Cys	[74]
F53	Patient				Yes		[106]
F54	Patient			M	Yes	Arg30stop	[107]
F55	Patient 1, B.G.	Greek	No	M	Yes	Arg73stop	[108]
F56	Patient 2, A.H.	German	Yes	M	Yes	c.1014C > G (p.V297PfsX6)	[108]
F57	Patient 3, A.J.	Indian	Yes	M	Yes	Gln259Pro	[108]
	Patient 4, U.A.	Indian	Yes	F	Yes	Gln259Pro	[108]
F58	Patient	Hmong		M	Yes	Phe251Cys	[109]
F59	Patient 1	Algerian	Yes	M	No	Trp286Arg	[110]
	Patient 2	Algerian	Yes	F	No	Trp286Arg	[110]
F60	Patient		Yes	M	No	Glu420Lys	[32]
F61	Patient	Iranian		F	Yes	Gln317stop	[111]
F62	Patient	Caucasian	No	F	Yes	Glu329Lys/c.489delC (p.Lys123SerX36)	[44]
F63	Patient	Saudi	Yes	M	No	Ile268Thr	[112]
F64	Patient	Saudi	Yes		Yes	Arg30stop	Unpublished
F65	FC	Arab	Yes	M	Yes	Tyr295stop	Unpublished
F66	Patient	Chilean		M	No	5 bp deletion 8 bp insertion	[113]
F67	Patient	Bedouin	Yes	M	Partial	Val346Met	[114]
F68	Patient	French	No	M	Yes	Leu263Arg/Arg391Ser	[115]
F69	Patient	Caucasian	Yes	M	Partial	Val26Met	[23]
F70	Patient	Czech/Indonesian				Arg30stop/c.859-861delCTT (ΔK246)	[22]
F71	Patient	Jamaican	?	M	Partial	102bp insertion duplication	[116]
F72	Patient	Hispanic	Yes	F	Partial	IVS8+1 (G > T)	[117]
F73	Patient 1	Brazilian		M	Yes	Gln259Asp	[118]
F74	Patient 2	Brazilian		M	Yes	Gly319Val	[118]
F75	Patient 3	Brazilian		F	Yes	Gln259Asp	[118]

F76	Patient 4	Brazilian	Yes	M		Arg73stop	[118]
F77	Patient	Iranian	Yes	M	Yes	Cys41Tyr	[119]
F78	Patient #1	Hispanic	Yes	F	Partial	Arg50stop	[24]
F79	Patient #2	Caucasian	?	F	Partial	Arg50stop	[24]
F80	JP	Sardinian	No	M	Partial	Cys84Arg	[120]
F81	Patient	Indian	Yes	M	Yes	c.716delA	[121]
F82	Patient	Thai			Partial	IVS4+1 G > C	[122]
F83		Korean		F		Arg158Cys/Thr146Ile	[123]
F84	Patient	Emariti	Yes	M	No	Arg274His	[124]
F85	Patient	Emariti	No	F	No	Arg274His	[124]
F86		German		F		Glu420Ala	[125]
F87	Patient 1	Saudi	Yes	M	No	Asp144Asn	[126]
F88		Chinese		F	No	His229Gln	[127]
F89				M		Arg30stop	[128]
F90		Egyptian			Yes	Arg30stop	[129]
F91		Egyptian			Yes	Tyr295stop	[129]
F92		Egyptian			Yes	Arg343Cys	[129]
F93		Egyptian			Yes	Arg391His	[129]
F94	Patient			M	No	Leu227Pro	[130]
F95	Patient 3	Macedonian	No		No	Arg158Cys	[126]
F96	Patient		No	F	Yes	Arg73stop	[131]
F97				F	Yes	Gly319Val	[128]
F98				M	Yes	Arg50stop	[132]
F99	Patient 3	Greek	No	F	Yes	IVS3+5 (G > C)	[133]
F100						c.147-2A > T	[134]
F101	Patient 4	Indian	Yes	F	Yes	delAG	[126]
F102						Arg73stop	[135]
F103	Patient			M	Yes	Gln152stop	[136]
F104		Greek			Yes	Gln356stop	[137]
F105	Patient 5	Turkish		M	Yes	Cys60Trp	[126]
F106	Patient 3	Chinese	No	M	No	Arg80Gln/N276Y	[126]

TABLE 68.2 Compilation of HVDRR cases.—cont'd

Family	Patient name/description	Ethnic origin	Parents related	Gender	Alopecia	VDR mutation	References
F107	Patient 1	Moroccan	Yes	M	Partial	Lys45Glu	[138]
	Patient 2			F	Partial	Lys45Glu	
F108	Z.H.	Kosovo	No	F	Partial		[139]
F109	Case 1	India/Muscat	Yes	M	Yes	Not determined	[140]
	Case 2			M	Yes		
	Case 3			F	Yes		
F110		Japanese	No	M	No	Gln400LeufsX7 Arg370His	[34]
F111	Patient	Chinese	No	F	Yes	Cys41Tyr	[141]
F112	Patient	Turkish		F	Yes	Ser360Pro	[142]
F113	Case 1	Turkish	Yes	F		Arg50stop	[143]
F114		Hispanic	No	F	No	Arg73Glu Arg274His	[144]
F115	Proband A.II.2	Lebanese	Yes	M	No	His397Pro	[145]
F116	Proband B.II.2	Lebanese	Yes	F	Partial	Arg391Ser	
F117	Proband C.II.1	Lebanese	Yes	M	Yes	Arg391Ser	
	Proband C.II.2			F	Yes	Arg391Ser	
F118	Proband	Taiwanese	No	M	Yes	Arg343His	[146]
F119	Case 1	Tunisian	Yes	M	Yes	Lys45Glu	[147]
	Case 2			M	Yes	Lys45Glu	
F120	Case 3		Yes	F	Yes	Lys45Glu	
F121	Case 4		No	F	Yes	Lys45Glu	
F122	Case 5		Yes	1	Yes	Lys45Glu	
F123	Case 6		Yes	F	No	Thr415Arg	
F124	Case 7		Yes	1	Yes	ND	
F125	Case 8		Yes	F	Yes	ND	
F126	Patient		Yes	F	Yes	Arg80Gln	[148]
F127	Patient 1	Saudi Arabian	Yes	F	Yes	Tyr295stop	[149]

	Patient 2			F		Tyr295stop	
	Patient 4			M		Tyr295stop	
	Patient 5					Tyr295stop	
	Patient 8					Tyr295stop	
F128	Patient 3	Saudi Arabian	Yes	F	Yes	Tyr295stop	
F129	Patient 6	Saudi Arabian	Yes	F	Yes	Tyr295stop	
F130	Patient 7	Saudi Arabian	Yes	M	Yes	Tyr295stop	
F131	Patient 1	Turkish	Yes	F	Yes	Gln152stop	[150]
F132	Patient 2	Turkish	Yes	M	Yes	Gln152stop	
F133	Patient 3	Turkish	Yes	F	Yes	c.756-2A > G	
F134	Patient 4	Turkish	Yes	M	Yes	c.66dupG (p.Ile23AspfsX20)	
F135	I-3, I-4		No	M	Yes	Gln152stop	[151]
F136	II-3		No	M	Yes	Arg73stop	
F137	III-5		No	M	No	Arg158Leu	
F138	IV-3		Yes	M	Yes	c.1-4A > G	
F139	V-3		Yes	F	Yes	c.755+1G > T	
F140	VI-3		No	M	No	Ile268Thr	
F141	VII-3		Yes	M	Yes	c.352delGACAG (p.Asp118SerfsX7)	
F142	VIII-5		Yes	M	Yes	Gln152stop	
F143	Patient	Iraqi	Yes	F	Yes	Arg80Trp	[152]
F144	Patient	Asian	Yes	M	Yes	Met52Thr c.1456delACCAAAG (p.Thr301ProfsX5)	[153]



found in the skin [40,154–156]. Feldman et al. [155] demonstrated that the VDR was present in fresh and cultured human foreskin as well as in cultured keratinocytes and dermal fibroblasts grown from adult skin biopsies. The studies to unravel the mutations in HVDRR patients began in earnest after those findings.

Eil et al. [69] showed in cultured skin fibroblasts that the disease was associated with defective nuclear uptake of 1,25(OH)<sub>2</sub>D, although the cause of this defect was not characterized (families F1 and F3). The following year, Feldman et al. [62] analyzed the VDR in cultured skin fibroblasts from two siblings with HVDRR (F11). The authors demonstrated that cytosolic extracts of cultured fibroblasts from these patients exhibited undetectable levels of [<sup>3</sup>H]1,25(OH)<sub>2</sub>D<sub>3</sub> binding. Furthermore, when cultured fibroblasts from normal subjects were treated with 1,25(OH)<sub>2</sub>D they were able to demonstrate an increase in 25-hydroxyvitamin D<sub>3</sub>-24-hydroxylase (24-hydroxylase) activity a well-characterized marker of 1,25(OH)<sub>2</sub>D responsiveness. However, the HVDRR patients' fibroblasts failed to induce 24-hydroxylase activity following treatment with high concentrations of 1,25(OH)<sub>2</sub>D due to defective VDR binding. Subsequently, a number of other HVDRR cases were examined using cultured skin fibroblasts [28,70,71,73,157] or cells derived from bone [83]. Some patients' fibroblasts lacked specific [<sup>3</sup>H]1,25(OH)<sub>2</sub>D binding [28,71,73,83,157] while other fibroblasts exhibited normal [<sup>3</sup>H]1,25(OH)<sub>2</sub>D binding but were nevertheless unresponsive to 1,25(OH)<sub>2</sub>D treatment suggesting defects beyond ligand binding [28,63,73,83,93,158]. Clemens et al. [157] on the other hand, showed that fibroblasts from HVDRR patients were resistant to 1,25(OH)<sub>2</sub>D by demonstrating a loss of the growth inhibitory effects of 1,25(OH)<sub>2</sub>D treatment in contrast to fibroblasts from healthy individuals that were growth-arrested. These early observations demonstrated that cells from HVDRR patients were resistant to 1,25(OH)<sub>2</sub>D and that a spectrum of abnormalities in the VDR could cause resistance. Many other patients with HVDRR have also been reported in the literature [51,78,84,139,140] and the details of these reports can be found in Table 68.2. The Adams et al. paper [84] compared HVDRR patients to those with XLH and the data suggested that VDR binding as well as postreceptor events are normal in patients with XLH.

As the number of reports on HVDRR increased, the heterogeneous nature of the defects in the VDR became more apparent. Hochberg et al. [37,38] reported the clinical findings in four patients from two unrelated families of Arab origin (F11,F18) who exhibited HVDRR and alopecia. A follow-up study by Chen et al. [64] showed that fibroblasts from three of these patients and a patient from an unrelated family from Germany (F17) had no detectable [<sup>3</sup>H]1,25(OH)<sub>2</sub>D-binding and 1,25(OH)<sub>2</sub>D

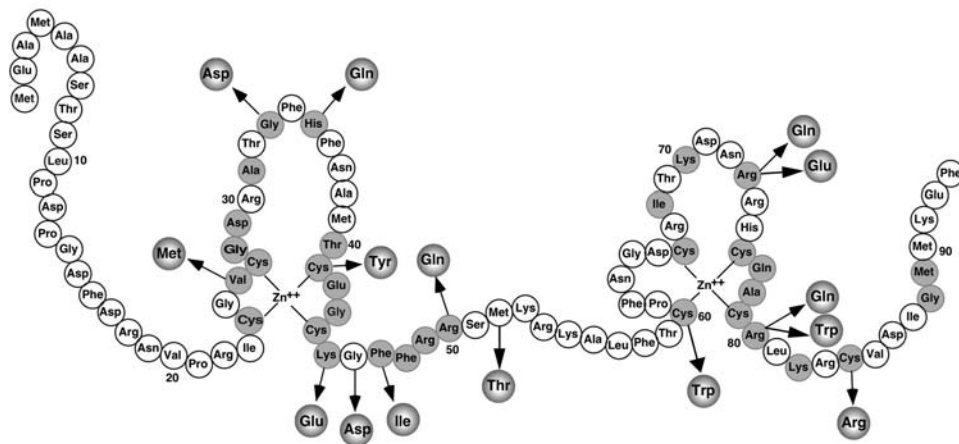
treatment failed to induce 24-hydroxylase activity. Pike et al. [159] used a radioligand immunoassay [160] and a monoclonal antibody to the chick VDR [161–163], to demonstrate the presence of an immunoreactive VDR protein in cell extracts from fibroblasts of HVDRR patients that exhibited no 1,25(OH)<sub>2</sub>D-binding. The authors speculated that the lack of [<sup>3</sup>H]1,25(OH)<sub>2</sub>D-binding in these patients was not due to defective synthesis of the VDR protein but was due to defects in the VDR LBD that abolished 1,25(OH)<sub>2</sub>D-binding [159]. Castells et al. [76] reported on a patient (F22) who had sparse hair, rickets, and high circulating 1,25(OH)<sub>2</sub>D levels. Studies of the VDR from the patient's fibroblasts showed that the VDR had decreased affinity for [<sup>3</sup>H]1,25(OH)<sub>2</sub>D. The patient showed a marked improvement after treatment with extremely high doses of 1,25(OH)<sub>2</sub>D apparently overcoming the low affinity binding abnormality in the LBD.

Based on the hypothesis that VDR binding to DNA was essential for its activity, Hirst et al. [63] examined whether defective VDR binding to DNA could be the cause of resistance in cases where the VDR exhibited normal ligand-binding. In a study of a family from Haiti (F19) with two sisters with clinical HVDRR and a normal unaffected sister, the authors showed that the fibroblasts from the two affected sisters had normal [<sup>3</sup>H]1,25(OH)<sub>2</sub>D-binding but were resistant to 1,25(OH)<sub>2</sub>D treatment. The authors further demonstrated that the VDR from the patient's fibroblasts exhibited a significant decrease in its affinity for DNA. A subsequent study by Malloy et al. [93] demonstrated a similar DNA binding defect in the VDR from HVDRR patients (F31) who had normal [<sup>3</sup>H]1,25(OH)<sub>2</sub>D binding. DNA-cellulose chromatography clearly revealed that the patient's VDR had a low affinity for DNA. Furthermore, the cells from the parents expressed two forms of the VDR, one with a high affinity for DNA and the other with a low affinity for DNA, the same as the affected daughters. This was the first evidence demonstrating the presence of the defective and normal VDR in cells from parents of HVDRR children. It was hypothesized that the VDR defects in these cases were likely be due to mutations in the VDR DBD [63,93], that later proved to be correct [65].

## 4.2 Studies in other cell types

A number of other cell types have been used to study the VDR in HVDRR patients. These include peripheral mononuclear cells [89], phytohemagglutinin (PHA)-stimulated lymphocytes [164,165], myeloid progenitor cells [90], Epstein–Barr virus (EBV) immortalized B lymphoblasts [33,65,66,93], and HTLV-1 virus immortalized T lymphoblasts [96]. It is interesting to note that although EBV immortalized B lymphoblasts from

### Mutations in the VDR DNA binding domain



**FIGURE 68.3** Model of the DNA-binding domain (DBD) of the VDR and location of mutations causing HVDRR. The two zinc-finger modules and the amino acid composition of the DBD are shown. Conserved amino acids are depicted as shaded circles. Numbers specify amino acid number.

normal subjects expressed wild-type VDR, they failed to induce 24-hydroxylase activity, and their growth was not inhibited by  $1,25(\text{OH})_2\text{D}$  [33]. A possible mechanism for these early findings was elucidated by Yenamandra et al. who showed that EBNA-3 a member of the EBNA-3-protein family that regulate transcription of cellular and viral genes, blocked the activation of VDR-dependent genes and protected the EBV immortalized lymphoblasts against vitamin- $\text{D}_3$ -induced growth arrest [166]. In contrast, PHA-stimulated lymphocytes and HTLV-1 immortalized T lymphoblasts from normal subjects do respond to  $1,25(\text{OH})_2\text{D}$  [96,167]. Takeda et al. [165] used PHA-stimulated lymphocytes from HVDRR patients to demonstrate  $1,25(\text{OH})_2\text{D}$  resistance by its failure to inhibit DNA synthesis or induce 24-hydroxylase activity [164,165]. Takeda et al. [165] also showed that PHA-stimulated lymphocytes from parents of children with HVDRR expressed intermediate levels of 24-hydroxylase in response to  $1,25(\text{OH})_2\text{D}$  compared to normal cells.

## 5. Mutations that cause HVDRR (VDDR-2A)

### 5.1 First description of a genetic defect in the nuclear receptor superfamily

The biochemical and cellular data obtained from the earlier studies of HVDRR patients provided a framework to begin the search for the specific molecular defect that inactivated the VDR and caused the disease. Investigations to determine the molecular nature of the mutations in the VDR that led to HVDRR began shortly after the human VDR cDNA sequence was elucidated by Baker et al.

[168]. During this same time period, the polymerase chain reaction (PCR) [169] was discovered that provided a method to amplify DNA for sequence analysis. The amino acid sequence of the VDR derived from the sequencing of the VDR cDNA suggested the presence of highly conserved zinc-finger structures that were thought to be involved in VDR binding to DNA. The initial sequencing studies focused on HVDRR patients with normal  $[^3\text{H}]1,25(\text{OH})_2\text{D}$  binding but abnormal binding to DNA since it was suspected that this defect would arise from mutations in the VDR DBD. In 1988, Hughes et al. [65] used PCR to amplify exons of the VDR gene from DNA isolated from the F19 and F31 families that were defective in DNA binding [63]. Patients in the F19 family were shown to have a unique G to A single base change in exon 3 that replaced arginine with glutamine at amino acid residue 73 (Arg73Gln) in the second zinc finger module of the DBD (Fig. 68.3). In the F31 family, a G to A transition was identified in exon 2 that changed glycine to aspartic acid at amino acid residue 33 in the first zinc finger module (Gly33Asp) (Fig. 68.3). The mutant sequences were only found in the children with HVDRR while their parents had a normal and mutant sequence demonstrating the genetic transmission and recessive nature of the disease. The study by Hughes et al. [65] was the first description of a genetic defect in a member of the steroid-thyroid-retinoid receptor gene superfamily. Mutations have now been found in many of the classical nuclear receptors including thyroid, androgen, estrogen, progesterone, glucocorticoid, and mineralocorticoid receptors.

To demonstrate that the Arg73Gln and the Gly33Asp mutations found in these families were the cause of

1,25(OH)<sub>2</sub>D resistance in the HVDRR patients, the mutations were recreated in the wild-type VDR cDNA by site-directed mutagenesis [67]. The mutant VDRs expressed in COS-1 cells exhibited normal [<sup>3</sup>H] 1,25(OH)<sub>2</sub>D-binding but weak binding to DNA similar to the VDR in the patient's fibroblasts. Importantly, in CV-1 cells, cotransfection with an osteocalcin-CAT reporter plasmid, showed that CAT activity could be induced by the wild-type VDR but not by the Arg73Gln or the Gly33Asp mutant VDRs. These data proved that the missense mutations caused the VDR to be transcriptionally inactive and were the cause of 1,25(OH)<sub>2</sub>D<sub>3</sub> resistance in the patients [67].

Since the original report of the genetic abnormality causing HVDRR by Hughes et al. [65] over 100 cases of HVDRR have been recorded and a number of these have been analyzed at the biochemical and molecular level (Table 68.2). Many different genetic abnormalities have now been identified in the VDR gene, including missense and nonsense mutations, but also deletions and splice site mutations. A description of these mutations and effects on VDR function is discussed below. Chapter 10 discusses in greater detail the structural defects in some of the HVDRR mutations and the mechanism by which the mutation impairs the function of the VDR to activate target genes.

## 5.2 Mutations in the VDR DNA binding domain (DBD)

Since the initial report by Hughes et al. [65], a number of mutations have been identified in the VDR DBD. The location of these mutations within the DBD is illustrated schematically in Fig. 68.3. Sone et al. [75] examined the VDR from two unrelated patients (F5 and F20) previously shown to exhibit a ligand-binding positive and low affinity DNA-binding phenotype by Liberman et al. [71,158]. In both patients, a mutation was identified that changed arginine to glutamine in the second zinc-finger module (Arg80Gln). The recreated Arg80Gln mutant VDR had a high affinity for binding [<sup>3</sup>H] 1,25(OH)<sub>2</sub>D, but had a lower affinity for DNA binding than WT DNA and was unable to activate gene transcription from a reporter plasmid demonstrating that this molecular defect was the cause of HVDRR in these cases [75]. Malloy et al. [102] also identified the same Arg80Gln mutation in two siblings with HVDRR (F49). The F49 family and the families (F5, F20) described by Sone et al. [75] both had origins in North Africa, however, no genetic relationship between these families could be established. This mutation was also identified in a female patient (F126) described by Nicolescu et al. [148].

Saijo et al. [49] found an Arg50Gln mutation in two unrelated families F26 and F33 while Yagi et al. [100]

identified a His35Gln mutation in family F45. Rut et al. [98] identified a Lys45Glu mutation in family F44 [99] and a Phe47Ile mutation in family F47 [101]. The Lys45Glu was subsequently found in seven patients in five families F107 [138], and F119, F120, F121, F122 [147].

Lin et al. [87] examined the VDR gene for mutations in a patient (F23) with HVDRR previously described by Sakati et al. [88]. A unique G to A base change resulted in a glycine being changed to aspartic acid at amino acid 46 (Gly46Asp) in the VDR DBD. In contrast to the other DBD mutations described above, the mutation at Gly46 occurs in an amino acid that is not well conserved in the steroid-thyroid-retinoid receptor superfamily. However, Gly46 is conserved among receptors that form heterodimers with RXR proteins such as thyroid receptor (TR) and retinoic acid (RAR) receptor. Additional unique missense mutations identified in the VDR DBD include a Val26Met mutation in family F69 [23], a Cys41Tyr mutation in families F77 and F111 [119,141], a Cys60Trp mutation in family F105 [126], an Arg80Trp mutation in family F143 [152], and a Cys84Arg mutation in family F80 [120].

The Lys45Glu and Gly46Asp mutations are located in the P-box (Fig. 68.2). This region of the VDR DBD is likely important in contacting the DNA bases and determining the specificity of the receptor for specific VDREs. Rut et al. [98] proposed that the Lys45Glu mutation would disturb the hydrogen bonding between Lys45 and a guanine nucleoside in the VDRE half-site and eliminate the ability of the VDR to interact with VDREs. The loss of the phenylalanine ring structure caused by the Phe47Ile mutation would disrupt the integrity of the hydrophobic core of the VDR DBD thereby altering binding to VDREs (Fig. 68.3) [98]. The Cys41Tyr and Cys60Trp mutations likely disrupt the coordination of the zinc ion and the formation of the zinc-finger structure. The Arg50Gln mutation occurs in the nuclear localization signal (NLS) that could inhibit the translocation of the VDR to the nucleus [54]. The Val26Met mutation likely disrupts the topography of the first zinc-finger module preventing binding to VDREs [23] while the Cys84Arg mutation may disrupt the alpha-helical structure of h-2 proposed at the base of the second zinc-finger module (Fig. 68.2). Arg80 directly forms hydrogen bonds with the backbone of the DNA in DNA-VDR complexes, and these hydrogen bonds were no longer present in DNA-VDR Arg80Trp complexes [152].

## 5.3 Mutations causing premature termination of the VDR

### 5.3.1 Nonsense mutations

Nonsense mutations in the VDR gene that introduce a premature stop signal cause early termination of the

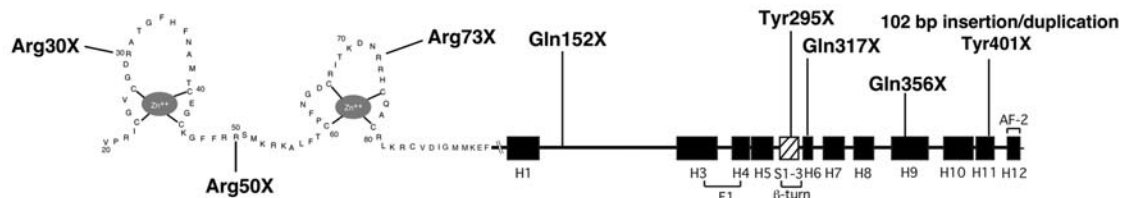
mature VDR protein. The first molecular analysis of three HVDRR cases (F18, F34, F36) [64,80,170] that exhibited no [ $^3\text{H}$ ]1,25(OH) $_2\text{D}$ -binding was reported by Ritchie et al. [66]. A single unique C to A base change was found in exon 7 that changed the codon for tyrosine (TAC) to an ochre termination codon (TAA) (Tyr295stop) (Fig. 68.4). The location of the premature stop at amino acid 295 truncates 132 amino acids of the carboxy terminus of the VDR that result in the deletion of a major portion of the LBD thereby creating the ligand-binding negative phenotype. The Tyr295stop mutation was the first nonsense mutation identified in the VDR. The location of this mutation and other nonsense mutations that cause premature termination of the VDR is shown in Fig. 68.4 and summarized in Table 68.2.

The three families studied by Ritchie et al. [66] and four additional families (F35, F37, F38, and F39) that comprised a large kindred in a region where consanguineous marriages were common were analyzed by Malloy et al. [33] (Table 68.2). A total of 8 children from this kindred exhibited HVDRR with alopecia. All of the affected children were homozygous for the Tyr295stop mutation and their parents were

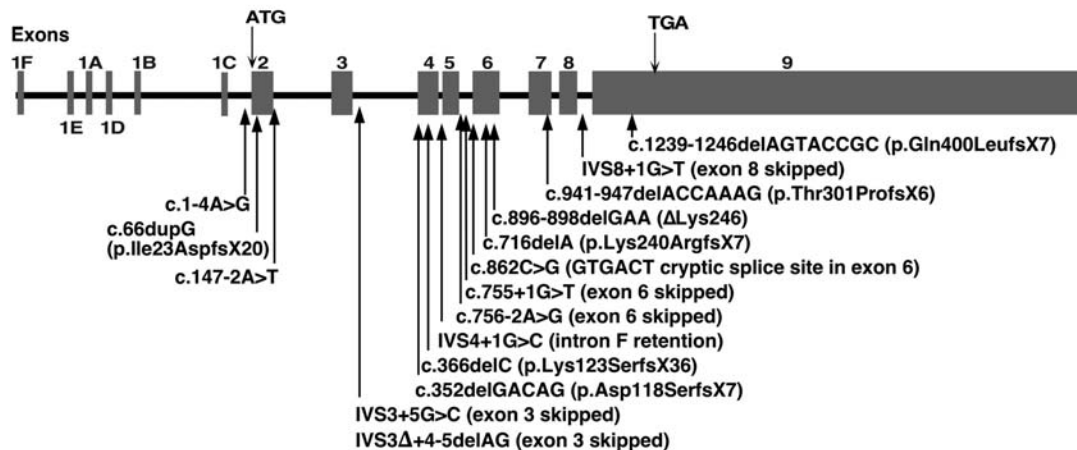
heterozygous. Interestingly, the 32,000 molecular weight truncated protein that is predicted to be produced by this mutation could not be detected in extracts of cultured dermal fibroblasts using Western blots. In addition, in all but one case, the VDR mRNA was undetectable on the Northern blot using RNA isolated from the cultured fibroblasts or EBV-transformed lymphoblasts from the patients. The absence of VDR mRNA indicated that the Tyr295stop mutation likely led to nonsense-mediated mRNA decay and, in this case, accounts for the absence of the mutant VDR protein [33]. This kindred is part of the Tiosano et al. cohort discussed later in Section 7.

The Tyr295stop mutation was also identified in patients (F29, F30) [92], in an Egyptian child (F91) [129], in two children in family F11 described in earlier papers [37,38,62,64,82] and in eight patients from four unrelated families with a history of consanguinity (F127, F128, F129, F130) [149]. A patient with HVDRR (F46) with no [ $^3\text{H}$ ]1,25(OH) $_2\text{D}$ -binding had a premature stop codon at amino acid 73 (Arg73stop) (Fig. 68.4) [92]. The Arg73-stop mutation was also identified in patients from families F55, F76, F96, and F102 [108,131,135]. Interestingly,

#### Stop mutations



#### Splicing mutations duplications deletions



**FIGURE 68.4** Splice site, duplication, and deletion mutations in the VDR. The locations of the nonsense mutations in the VDR protein shown (top figure). Lower figure shows the location of splice site and duplications/deletions mutations in the VDR gene.



the patient from family F96 was a 2-year-old girl with alopecia, short stature, and rickets without a family history of consanguinity. The child was homozygous for the nonsense mutation that caused the Arg73stop. Her mother was heterozygous for the mutation, but her father was negative. The authors excluded gross deletion of the father's allele or paternal discordance. Genome-wide SNP array of the family (the patient and her parents) showed complete maternal isodisomy of chromosome 12. The patient was successfully treated with high-dose oral calcium. This was the first reported case of Uniparental Disomy involving chromosome 12 [131].

A number of other premature stop mutations have been identified in the VDR and the locations of these mutations are shown in Fig. 68.4. The premature stop mutations causing HVDRR include an Arg30stop mutation in families F53 [106], F54 [107], F64, F89 [128], and F90 [129], and a Gln152stop mutation in families F32 [72,92], F103 [136], F131 and F132 [150], F135 and F142 [151]. Interestingly, the parents in family F32 who were first cousins and heterozygous for the Gln152stop mutation had elevated levels of serum 1,25(OH)<sub>2</sub>D. The mean value for the father was 73 pg/mL and for the mother 93 pg/mL (normal range 20–80 pg/mL). The elevated 1,25(OH)<sub>2</sub>D values raise the possibility of mild vitamin D resistance in the heterozygotic parents, a finding that had not been documented previously in other parents of HVDRR children. A young Iranian girl (F61) with HVDRR and alopecia was reported by Bouillon and Vainsel [171]. The patient's fibroblasts expressed the VDR mRNA but had no VDR protein and were unresponsive to high doses of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Sequence analysis of the VDR gene uncovered a glutamine being replaced by a termination codon (Gln317stop) (Fig. 68.4) [111]. Forghani et al. [24] studied two unrelated patients (F78, F79) with HVDRR and alopecia living in different parts of the USA. Both patients had a mutation that changed arginine at amino acid 50 to a stop codon (Arg50stop). The Arg50stop mutation was also found in families F98 and F113 [132]. Two Greek patients (F104) with HVDRR and alopecia had a mutation that changed glutamine at amino acid 356 to a stop codon (Gln356stop) [137].

### 5.3.2 Mutations that disrupt mRNA splicing

Hawa et al. [103] examined the VDR in a young Greek girl (F50) with HVDRR and alopecia. They showed that the patient's RNA sequence diverged from the wild-type sequence at nucleotide 147. The sequence from exon 3 that encodes the second zinc finger module was deleted and the sequence that followed was from exon 4. Sequence analysis of the VDR chromosomal gene found no mutations in the exons, however, a G to C base change was found in the 5' end of intron E (Fig. 68.4). This single nucleotide change converted the

wild-type sequence from GTGAGT to GTGACT and eliminated the 5' donor splice site (IVS3+5G > C). The loss of the 5' donor splice site caused exon 3 to be skipped causing a reading frameshift that resulted in a premature stop codon. The mutant protein contained 92 amino acids of the wild-type sequence plus 6 amino acids due to the frameshift (Glu92fsX7). This mutation was also found in a patient from family F99 [133].

A young Thai girl (F82) with partial alopecia had a single base substitution in the 5'-donor splice site at the exon 4-intron F junction [122]. The mutation caused 254 base pairs of intron F to be incorporated into the VDR mRNA (IVS4+1G > C). The encoded VDR protein contained 154 amino acids of the wild-type sequence and an additional 23 amino acids from the unspliced intron F. A splice site mutation was also identified in a young Hispanic girl (F72) with HVDRR and partial alopecia. A novel G to T substitution was identified in the 5'-splice site in the exon 8-intron J junction (IVS8+1G > T) [117]. Sequence analysis showed that exon 8 was skipped and that exons 7 and 9 were fused in the frame. However, the mutation caused a 39 amino acid deletion ( $\Delta$ Ala303-Pro341) in the VDR LBD. The recreated mutant VDR $\Delta$ Ala303-Pro341 was unable to activate gene transcription (Malloy unpublished study). A young child (F100) with HVDRR and alopecia was shown to have an A to T substitution that caused a splice site mutation (c.147-2A > T) [134].

Other splice site mutations have been identified (Fig. 68.4). Cockerill et al. [108] described a patient (F56) with a C to G mutation at nucleotide position 702 that generated a cryptic 5' splice site leading to the removal of part of exon 7. The mutant protein contained 233 amino acids of the wild-type sequence and an additional 4 amino acids due to a frameshift (Leu233fsX5). A young girl (F133) from Turkey had a novel mutation in the consensus splice acceptor site (c.756-2A > G), that resulted in abnormal splicing [150]. Demir et al. [151] identified two splice site mutations in the VDR gene. In one patient (F138) a novel c.1-4A > G mutation located in the canonical splice acceptor site of intron 3 and was predicted to cause the skipping of exon 2, which contains the first coding exon of the VDR gene. In a second patient (F139) a c.755+1G > T mutation located in the canonical splice donor site of intron 8 and was predicted to cause exon 8 skipping.

### 5.3.3 Deletions

Deletion mutations are shown in Fig. 68.4. In a preliminary report of patient (F42), a major structural defect in the VDR gene was described as the cause of HVDRR [97]. The defect, found by PCR and Southern blotting, was a deletion in the VDR gene that eliminated exons 7–9. A young girl from India with HVDRR and alopecia was found to have a one bp deletion in exon 6



(c.716delA) [121]. The mutation caused a shift in the reading frame that introduced a premature stop codon and resulted in the truncation of the VDR LBD (p.Lys240ArgfsX7). A second girl (F101) from India had a 2 bp (AG) deletion in the 5'-donor splice site at the exon 3-intron E boundary (IVS3Δ+4-5 del AG) [126]. The mutation likely caused exon 3 skipping. One patient (F141) was shown to have a five base deletion (c.352delGACAG) resulting in an Asp118Ser mutation and frameshift resulting in a premature stop codon seven amino acid downstream (p.Asp118SerfsX7) [151]. All of the nonsense mutations and all splice site mutations that lead to frameshifts, as well as the gene deletion mutations result in truncated VDRs. Although some of the mutant VDRs may have intact DBDs, the loss of the LBD and therefore its ability to bind 1,25(OH)<sub>2</sub>D, or associate with RXR, and interact with coactivators makes the mutant receptors nonfunctional and causes complete hormone resistance.

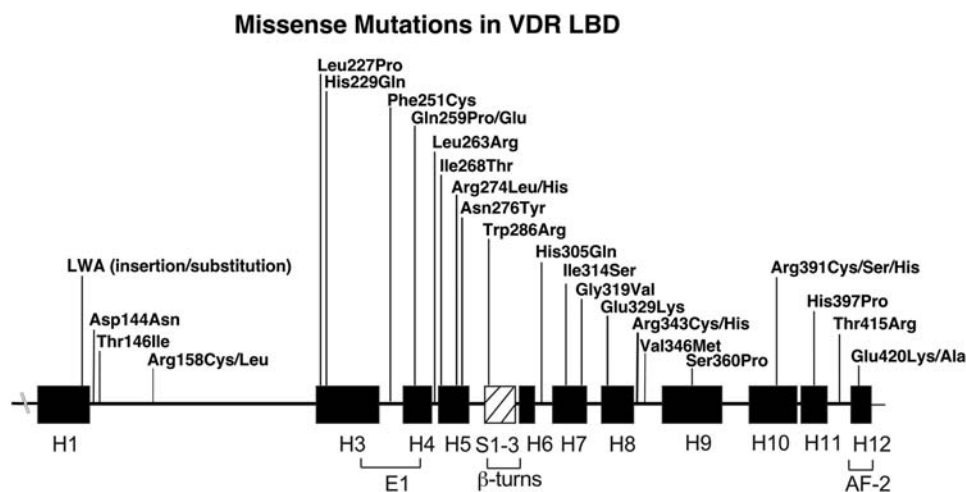
## 5.4 Mutations in the VDR ligand-binding domain (LBD)

### 5.4.1 Mutations that affect 1,25(OH)<sub>2</sub>D<sub>3</sub>-binding

LBD mutations are illustrated in Fig. 68.5. Rut et al. [85] and Kristjansson et al. [86] identified the first missense mutation in the VDR LBD. The patient from Kuwait (F21) had HVDRR but did not have alopecia. Preliminary studies by Fraher et al. [31] on the patient's fibroblasts showed absent [<sup>3</sup>H]1,25(OH)<sub>2</sub>D<sub>3</sub>-binding. However, Rut et al. [85] later showed that the fibroblasts contained normal amounts of [<sup>3</sup>H]1,25(OH)<sub>2</sub>D-binding but the affinity of the receptor for 1,25(OH)<sub>2</sub>D was significantly reduced ( $K_d$   $1 \times 10^{-9}$  M) compared to normal controls ( $K_d$   $0.7 \times 10^{-10}$  M). They showed that the

patient's fibroblasts were resistant to vitamin D by their failure to induce 24-hydroxylase activity when treated with high doses of 1,25(OH)<sub>2</sub>D [31,85]. Molecular analysis of the VDR gene identified a unique G to T missense mutation in exon 7 that replaced arginine residue with leucine at amino acid 274 (Arg274Leu) (Fig. 68.5) [85,86]. As described by Rochel et al. [55] and discussed in Chapter 10, Arg274 is a contact point for the 1 $\alpha$ -hydroxyl group in 1,25(OH)<sub>2</sub>D. In transactivation assays, the recreated Arg274Leu mutant VDR was relatively resistant to vitamin D requiring approximately 1000-fold more 1,25(OH)<sub>2</sub>D to activate gene transcription than the wild-type receptor [86]. Although the resistance caused by the defective VDR could be overcome by treating with high concentrations of 1,25(OH)<sub>2</sub>D in vitro, the patient failed to respond to massive doses of the hormone and eventually died of pneumonia.

An Arg274His mutation was identified in two unrelated children (F84, F85) from the United Arab Emirates with HVDRR without alopecia by Aljubeih et al. [124]. In transactivation assays, both the Arg274Leu and Arg274His mutants showed ~100-fold lower responsiveness to 1,25(OH)<sub>2</sub>D compared to the WT VDR. Whitfield et al. [74] analyzed the VDR from a girl (F4) who had the classic symptoms of HVDRR but without alopecia. The patient's fibroblasts had normal 1,25(OH)<sub>2</sub>D<sub>3</sub> binding but had defective induction of 24-hydroxylase activity [73]. Sequencing of the VDR gene uncovered a T to G substitution in exon 8 that changed isoleucine to serine at amino acid 314 (Ile314Ser) (Fig. 68.5). The Ile314Ser mutation caused a subtle defect in RXR heterodimerization and decreased response to 1,25(OH)<sub>2</sub>D in vitro [74]. Interestingly, the patient showed a nearly complete cure of rickets when treated with pharmacological doses of 25(OH)D.



**FIGURE 68.5** Schematic illustration of the ligand-binding domain (LBD) of the VDR and location of missense mutations that cause HVDRR. The  $\alpha$ -helices (H1–H12) of the VDR LBD are depicted as shaded rectangles and the  $\beta$ -turns are drawn as a hatched rectangle. The loops connecting the helices are drawn as solid lines. The E1 and AF-2 regions are shown below the  $\alpha$ -helices.

A His305Gln missense mutation in the VDR LBD was described by Malloy et al. [104]. The patient a Turkish boy (F51), had HVDRR and two other rare disorders, congenital generalized lipomatrophic diabetes (Berardinelli-Seip syndrome), and Persistent Müllerian Duct Syndrome (PMDS) [105]. The patient had rickets and elevated 1,25(OH)<sub>2</sub>D levels but did not have alopecia. He was treated with extremely high doses of calcitriol (Rocaltrol 12.5 µg/day) that eventually normalized his serum calcium and ultimately improved his rickets. However, the child died of apparently unrelated problems. In vitro the patient's fibroblasts expressed normal VDR levels but the affinity for 1,25(OH)<sub>2</sub>D was decreased by about twofold. The patient's fibroblasts required approximately a fivefold more 1,25(OH)<sub>2</sub>D to induce *CYP24A1* mRNA compared to control cells. In gene transactivation assays, the His305Gln mutant VDR required fivefold more 1,25(OH)<sub>2</sub>D to achieve the same level of activity as the wild type VDR. The boy's sister, who also had HVDRR and the same mutation in the VDR, did not exhibit the other two genetic defects her brother had. No explanation was forthcoming for the presence of three genetic defects in a single individual. It is unclear how the His305Gln mutation in the VDR is related, if at all, to the two other genetic abnormalities that were present in this child. The congenital total lipodystrophy was subsequently shown to be due to a mutation in the seipin gene (*BSCL2*) [172]. While a genetic cause for the PMDS was not determined, Malloy et al. have shown that Müllerian-inhibiting substance (MIS) is regulated by the VDR [173]. Since MIS initiates the regression of the Müllerian ducts that in a normal female embryo develop into the uterus, fallopian tubes, and upper vagina, and in males also controls the induction of the abdominal phase of testicular descent [174]. One might speculate that PMDS was due to the VDR mutation causing lack of Müllerian duct regression in this child. However, the fact that PMDS has not been reported in other children with inactivating VDR mutations, or in the sister with the same mutation, makes it unlikely to be the cause.

Other LBD mutations that affect 1,25(OH)<sub>2</sub>D binding are shown in Fig. 68.5. An Algerian boy and his younger sister (F59) both with HVDRR without alopecia were shown to have a Trp286Arg mutation (Fig. 68.5) [110]. A young Saudi Arabian girl (F63) with HVDRR but without alopecia was found to have a Ile268Thr mutation [112]. The Ile268Thr mutant VDR exhibited a ~10-fold lower affinity for [<sup>3</sup>H]1,25(OH)<sub>2</sub>D binding compared to the WT VDR. However, in transactivation assays, the Ile268Thr mutant required ~100-fold higher concentrations of 1,25(OH)<sub>2</sub>D to stimulate gene transcription compared to the WT VDR. The Ile268Thr mutation was also identified in a boy (F140) with HVDRR

without alopecia [151]. A Saudi Arabian boy with HVDRR without alopecia was shown to have an Asp144Asn mutation in the VDR LBD [126]. In transactivation assays, the recreated Asp144Asn mutant was ~33-fold less responsive to 1,25(OH)<sub>2</sub>D compared to the WT VDR. A young boy (F95) from Macedonia with HVDRR without alopecia was found to have an Arg158Cys mutation in the VDR LBD [126]. The patient's fibroblasts exhibited a minimal response to 100 nM 1,25(OH)<sub>2</sub>D. An Arg158Leu mutation was also identified in a boy (F137) with HVDRR without alopecia [151].

A young boy (F94) with HVDRR without alopecia was found to have a Leu227Pro mutation in the LBD [130]. In transactivation assays the recreated Leu227Pro mutant exhibited little to no activity when treated with 1 µM 1,25(OH)<sub>2</sub>D. A Turkish girl (F112) with HVDRR with alopecia was shown to have a Ser360Pro mutation in the VDR LBD. Functional studies showed that the Ser360Pro mutant had no [<sup>3</sup>H]1,25(OH)<sub>2</sub>D binding and failed to interact with RXR and coactivators SRC-1 and N-CoR [142]. The detailed structural analysis of some LBD and other VDR mutations that affect 1,25(OH)<sub>2</sub>D binding or activity is further discussed in Chapter 10.

#### 5.4.2 Mutations that affect VDR-RXR heterodimerization

As discussed previously, the VDR requires heterodimerization with RXR for gene regulation activity. Disruption of the protein:protein interaction with RXR can thereby cause 1,25(OH)<sub>2</sub>D resistance. These mutations are illustrated in Fig. 68.5. The first patient found to have a mutation in the VDR that disrupted VDR-RXR heterodimerization was described by Whitfield et al. [74]. The patient a young girl (F52) with HVDRR and alopecia, was found to have a mutation that changed arginine to cysteine at amino acid 391 (Arg391Cys). Ligand binding was normal but transactivation of a reporter gene by the Arg391Cys mutant VDR required higher than normal concentrations of 1,25(OH)<sub>2</sub>D for activity. However, when RXR was cotransfected in the assays the transactivation activity could be restored to normal levels. In electrophoretic mobility shift assays (EMSA) the Arg391Cys mutant exhibited a lower capacity for forming a VDR-RXR-VDRE complex than the wild-type VDR. This study demonstrated the importance of both 1,25(OH)<sub>2</sub>D-binding and RXR heterodimerization in VDR-mediated gene transactivation. The fact that the girl had alopecia was instructive in that disruption of VDR-RXR dimerization, can cause alopecia. This finding is also noted in the following cases substantiating the point.

An Arg391His mutation was identified in an Egyptian child (F93) [129] and an Arg391Ser (F116, F117) was found in three Lebanese patients from two families

(F116, F117) [145]. Like the patient with the Arg391Cys mutation, these children also had alopecia. Two siblings, a brother and sister from India (F57) that had HVDRR with alopecia, had a missense mutation that changed glutamine to proline at amino acid 259 (Gln259Pro) [108] (Fig. 68.5). Although Gln259Pro had no apparent effect on ligand binding there was evidence of impaired VDR-RXR-VDRE formation. In two Brazilian patients (F73, F75) with HVDRR and alopecia, a missense mutation was found that changed Gln259 to aspartic acid (Gln259Asp) [118]. Like the Gln259Pro mutation, the Gln259Asp mutation would also be predicted to affect heterodimerization with RXR.

Malloy et al. [109] examined the VDR in a young Hmong boy (F58) with HVDRR and alopecia. A unique missense mutation was found that changed a phenylalanine to cysteine at amino acid 251 (Phe251Cys) (Fig. 68.5). In [ $^3\text{H}$ ]1,25(OH) $_2\text{D}_3$  binding experiments, the recreated Phe251Cys mutant VDR exhibited a lower affinity for the ligand than wild-type VDR. In GST pull-down assays and yeast two-hybrid assays, the Phe251Cys mutant VDR was shown to have a reduced capacity to bind RXR. In transactivation assays, co-transfection of RXR partially restored the activity of the mutant receptor. The Phe251Cys mutation occurs in the E1 region (amino acids 244–263) of the VDR LBD. The E1 region overlaps the C-terminal portion of helix H3, loop 3-4 and the N-terminal portion of helix H4.

Two patients (F74, F97) with HVDRR and alopecia were shown to have a mutation that changed glycine to valine at amino acid 319 (Gly319Val) [118,128]. Damiani et al. [128] noted that their patient had normal bone mass and normocalcemia in adulthood despite homozygous vitamin D receptor mutations. Gly319 is part of the dimerization interface (residues 317–325) of the VDR that interacts with RXR [175]. A Val346Met mutation was identified in Bedouin boy with HVDRR and alopecia [114]. The patient also had papular lesions on the scalp and face. Two brothers and one sister were also affected. The affected children also had mild hearing loss and secondary speech abnormalities. Although hearing loss had not been noted before in other HVDRR patients, progressive hearing loss was found in VDR knockout mice [176].

#### 5.4.3 Mutations that affect coactivator-binding

As noted above, the VDR also must recruit coactivator proteins for transcriptional activity. It is now clear from crystallographic studies of the VDR and other members of the steroid receptor superfamily that repositioning of helix H12 is an essential event that occurs as a consequence of ligand binding and is required for transactivation [55]. The repositioning of helix 12 leads to the formation of the hydrophobic cleft critical for coactivator

binding. Therefore, disruption of this surface interface may cause hormone resistance and HVDRR.

A study by Malloy et al. [32] examined the VDR in a young boy (F60) with HVDRR without alopecia. The patient's fibroblasts exhibited normal ligand binding but the cells were totally resistant to 1,25(OH) $_2\text{D}$  action. A novel missense mutation was found in exon 9 that changed a glutamic acid to lysine at amino acid 420 (Glu420Lys) (Fig. 68.5). The Glu420Lys mutation is located in helix H12. The recreated Glu420Lys mutant VDR showed no defects in VDR-RXR heterodimerization or binding to VDREs. However, the mutation prevented the coactivators SRC-1 and DRIP205 from binding to the VDR. In transactivation assays, cotransfection of SRC-1 failed to restore transactivation by the mutant VDR. This case represented the first description of a naturally occurring mutation in the VDR that disrupts coactivator interactions and causes HVDRR [32]. The polar interactions that stabilize the repositioning of helix H12 involve a conserved salt-bridge between Lys264 in helix H4 and Glu420 in helix H12 and a hydrogen bond between Ser235 in helix H3 and Thr415 in helix H12 [55]. The substitution of the negatively charged glutamic acid (Glu420) with a positively charged lysine (Lys420) would prevent the polar interaction with the positively charged lysine (Lys264) salt bridge partner. The charge clamp formed by Lys264 and Glu420 enables the VDR to recruit and bind coactivators through their LxxLL motifs. The Glu420Lys mutation prevents the correct repositioning of helix H12 after binding the ligand that disrupts coactivator binding and causes the hormone resistance seen in the patient.

An Egyptian child (F92) with HVDRR and alopecia was found to have a mutation that changed arginine to cysteine at amino acid 343 (Arg343Cys) [129]. A second mutation in the Arg343 residue (Arg343His) was identified in a patient (F118) with alopecia [146]. Interestingly, Arg343 resides in helix H9 and is important in RXR heterodimerization. However, functional analysis of the Arg343His mutant VDR showed no apparent defect in RXR heterodimerization. Modeling studies indicate that the Arg343 residue contacts Glu269 of the H5 structure of VDR, which is important in the formation of a hydrophobic cleft with H3 and H4 that forms binding sites for transcriptional coactivators and co-repressor molecules. In support of this, the addition of GRIP-1 did not rescue transactivation by the Arg343His mutant VDR. These results suggest that mutations that cause instability of the H5 structure of VDR can result in alopecia.

A Lebanese boy (F115) without alopecia was shown to have a His397Pro mutation [145]. The patient later presented with multi-lobar pneumonia and died of cardiopulmonary arrest. His397 is involved in stabilizing



helix H12 positioning and thus the His397Pro mutation likely disrupts coactivator binding. A patient (F123) without alopecia was found to have a Thr415Arg mutation [147]. Thr415 is also involved in stabilizing helix H12 positioning and the Thr415Arg mutation likely disrupts coactivator binding.

## 5.5 Compound heterozygous mutations in the VDR

There are several reports of patients with HVDRR with compound heterozygous mutations in the VDR gene. There is no consanguinity in these patient's families and the asymptomatic parents each harbor a mutation in one allele of their VDR gene that is passed to their offspring. In patient (F62) with HVDRR and alopecia a heterozygous missense mutation was found that caused a Glu329Lys substitution (Fig. 68.5) [44]. The patient was also heterozygous for a mutation in exon 4 that deleted a single cytosine at nucleotide 366 (c.366delC). The c.366delC deletion resulted in a frameshift that led to a premature termination signal that truncated the VDR (p.Lys123SerfsX36) (Fig. 68.4).

Compound heterozygous mutations were also identified in the VDR gene in a child (F68) with HVDRR, total alopecia, and early childhood-onset type 1 diabetes [115]. A missense mutation was found in exon 7 that caused a Leu263Arg substitution in helix H4. A second missense mutation was found in exon 9 that caused an Arg391Ser substitution in helix H10. As described above, an Arg391Cys mutation was previously shown to affect RXR heterodimerization thus it was likely that the arginine to serine substitution also affected heterodimerization [74]. Interestingly, the Leu263Arg mutant and the Arg391Ser mutant VDRs exhibited differential activity on 24-hydroxylase and RelB promoters. The 24-hydroxylase responses were abolished in the Leu263Arg mutant but only partially altered in the Arg391Ser mutant. On the other hand, RelB responses were normal for the Leu263Arg mutant but the Arg391Ser mutant was defective in this response [115].

Compound heterozygous mutations were also identified in a young girl (F70) with HVDRR and alopecia [22]. One mutation changed the codon for arginine to a nonsense mutation at amino acid 30 (Arg30stop). The second mutation was an in-frame 3-bp deletion in exon 6. The 3-bp deletion (c.896-898delGAA) removed the codon for lysine (GAA) at amino acid 246 ( $\Delta$ K246) but did not alter the reading frame (Fig. 68.4). The patient's fibroblasts expressed the VDR $\Delta$ K246 mutant protein but were unresponsive to 1,25(OH) $_2$ D. The  $\Delta$ K246 mutation abolished heterodimerization with RXR and binding to coactivators.

A Korean girl (F83) with HVDRR without alopecia was heterozygous for two VDR mutations [123]. The girl and her father were both heterozygous for a C-to-T mutation in exon 4 that changed a threonine to isoleucine at amino acid 146 (Thr146Ile). The girl and her mother were both heterozygous for C-to-T mutation in exon 5 that changed arginine to cysteine (Arg158Cys).

A Chinese boy (F106) with HVDRR without alopecia was heterozygous for two VDR mutations [126]. The boy and his mother were heterozygous for a mutation in exon 3 that changed arginine to glutamine at amino acid 80 (Arg80Gln). The patient was also heterozygous for an A to T base substitution in exon 7 that changed asparagine to tyrosine at amino acid 276 (Asn276Tyr). Interestingly, the father had no mutations in his VDR gene suggesting that the Asn276Tyr mutation arose spontaneously. The Arg80Gln mutation was previously found in three families all with origins in North Africa [75,102].

A Chinese girl (F88) with HVDRR without alopecia had a heterozygous C to G substitution in exon 6 that changed histidine to glutamine at amino acid 229 (His229Gln) [127]. The patient also was heterozygous for the Met1 and Met4 polymorphisms in the VDR translation start sites. Sequencing of the patient's VDR cDNA showed that the Met1 and Met4 start sites were both present on the allele that had the His229Gln mutation. This meant that the allele with the His229 wild-type sequence had the Met1 and Met4 polymorphisms that did not encode a start site. A potential downstream start site at Met40 would have deleted the first zinc finger of the DBD rendering this protein inactive. An Asian boy (F144) was found to have two heterozygous mutations in the VDR gene. One mutation changed a methionine to threonine at amino acid 52 (Met52Thr). The second mutation was a seven bp deletion (c.941-947delACCAAAG) that caused a frameshift (Thr301ProfsX6) that truncated the VDR [153].

## 5.6 Dominant negative mutations

A mutation in the Glu420 residue was found in a German girl (F86) with HVDRR without alopecia. In this case, glutamic acid was mutated to alanine (Glu420Ala) [125]. The girl and her father were heterozygous for the missense mutation while the mother had no mutations in the VDR gene. The girl's father showed minor symptoms of vitamin D resistance. Fibroblasts from the girl and her father showed resistance to 1,25(OH) $_2$ D $_3$ . The heterozygous Glu420Ala mutation appears to act in a dominant negative manner silencing the WT VDR in the girl and possibly also causing bone disease and resistance to vitamin D action in the father. This

was the first report of HVDRR caused by a heterozygous missense mutation.

A Japanese boy (F110) with short stature and bowlegs was shown to have an 8 base pair deletion (c.1239-1246delAGTACCGC) and an Arg370His substitution [34]. The Arg370His mutant VDR appeared to have no effect on transcriptional activity. The 8 base pair deletion resulted in a frameshift (p.Gln400LeufsX7) that deleted helix H12. The Gln400LeufsX7 truncated VDR was transcriptionally inactive but exhibited a dominant-negative effect on wild-type VDR in transactivation assays [34]. The boy's father who was heterozygous for the Gln400LeufsX7 mutation also had slight bowing of legs. A Hispanic female (F114) with HVDRR without alopecia was shown to be heterozygous for both Arg73Glu and Arg274His mutations [144].

### 5.7 Other mutations in the VDR

A young boy (F66) from Chile with HVDRR without alopecia was found to have a unique 5-bp deletion/8-bp insertion in exon 4 of the *VDR* gene [113]. The mutation in helix H1 of the LBD deleted His141 and Tyr142 and inserted three amino acids in their place (Leu141, Trp142, and Ala143). The patient's fibroblasts had no demonstrable [ $^3\text{H}$ ]1,25(OH) $_2$ D binding and were resistant to 1,25(OH) $_2$ D treatment. When the effects of the three individual mutations were analyzed separately, only the insertion of Ala143 into the WT VDR disrupted VDR transactivation to the same extent observed with the child's mutated VDR. A young Jamaican boy (F71) with HVDRR and patchy alopecia was found to have a 102 bp insertion/duplication in the *VDR* gene that introduced a premature stop (Tyr401stop) [116]. The mutation deleted a part of helix H11 and all of helix H12. The patient's fibroblasts expressed the truncated VDR but were resistant to 1,25(OH) $_2$ D. The truncated VDR weakly bound [ $^3\text{H}$ ]1,25(OH) $_2$ D and was able to heterodimerize with RXR, bind to DNA and interact with the corepressor hairless (HR). However, the truncated VDR failed to bind coactivators and was transactivation defective. A Turkish patient (F134) with HVDRR and alopecia was found to have a duplication of guanine (c.66dupG) in exon 2. The mutation causes a frameshift (p.Ile23AspfsX20) that truncates the VDR [150].

### 5.8 HVDRR in dogs and cats

There are several reports of HVDRR in pets. HVDRR was diagnosed in a Pomeranian dog [177]. On physical examination, the dog exhibited lateral bowing of the antebrachium of both forelimbs and generalized non-pruritic partial alopecia. Serum biochemistry revealed marked hypocalcemia and increased alkaline

phosphatase. Although her alopecia was resolving, the dog's coat was of poorer quality than that of her half-sibling. At 8 months of age, while receiving 1,25(OH) $_2$ D, the dog's serum calcium was very low (total calcium 5.4 mg/dL; normal 8.9–11.4 mg/dL) despite exceptionally high 25(OH)D levels (1017 pg/mL; normal 25–50 pg/mL) indicative of HVDRR. Analysis of the *VDR* gene revealed a single base deletion in exon 4 that led to a frameshift causing the amino acid sequence to diverge after Arg175. An additional 41 amino acids were in the frame before a downstream termination signal occurred. The mutation deleted a major part of the LBD and caused the VDR to be nonfunctional. The dog was eventually treated with intravenous (IV) calcium gluconate. After initial stabilization, a neurologic examination revealed signs attributable to diffuse forebrain disease and hindlimb paresis. Spinal radiographs and computed tomography confirmed a fracture of T11 and showed accompanying spinal cord compression. Flaring of the ribs at the costochondral junctions (so-called rachitic rosary in people) was also readily apparent on radiographs. Given the severity of the dog's spinal injury, the decision was made to euthanize the animal. This was the first molecular description of a mutation causing HVDRR in a mammal other than humans.

Two cats reported earlier had the classic picture of HVDRR but any *VDR* mutations were not described. In one report, a 4-month-old male domestic shorthair cat was examined because of lethargy, vomiting, diarrhea, muscle tremors, and mydriasis. Laboratory evaluation revealed hypocalcemia, hypophosphatemia, and high serum 1,25(OH) $_2$ D and PTH levels. The cat was treated with oral calcium and calcitriol supplements. After 18 months of treatment, the cat was clinically normal. After the treatment was discontinued, the cat was able to maintain normal serum calcium levels [178]. This is reminiscent of some HVDRR patients after puberty.

A second report described the biochemical abnormalities and radiographic changes in a 4-month-old kitten with symptoms of HVDRR [179]. The kitten was treated with calcium and vitamin D. The treatment failed to increase the serum calcium levels or reverse the lateral antebrachial bowing, lumbar spinal lordosis and costochondral beading. The cat was euthanized at 9 years of age as a result of refractory hip pain [180].

### 5.9 HVDRR without mutations in the VDR

Since the initial description of HVDRR as a genetic disorder, mutations in the *VDR* gene were suspected as the likely cause of 1,25(OH) $_2$ D resistance. Although the *VDR* is the principal determinant of the 1,25(OH) $_2$ D action pathway, it is possible that target



organ resistance to  $1,25(\text{OH})_2\text{D}$  may result from mutations in other proteins that are essential to the transactivation process.

Hewison et al. [16] have described a case of HVDRR in which a mutation could not be found in the VDR. The patient (F48), a young girl of English descent, exhibited all of the hallmarks of HVDRR including alopecia. The patient's fibroblasts expressed a normal-sized VDR mRNA and exhibited normal  $[^3\text{H}]1,25(\text{OH})_2\text{D}$  binding. However, no induction of 24-hydroxylase activity was observed when the fibroblasts were treated with up to  $1\ \mu\text{M}$   $1,25(\text{OH})_2\text{D}$ . The patient's VDR mRNA was reverse-transcribed and amplified by PCR however, no mutations were found in the VDR cDNA. In transactivation assays, the patient's VDR cDNA exhibited a normal transactivation response to  $1,25(\text{OH})_2\text{D}$  in CV-1 cells. These results indicated that the patient's VDR DNA sequence was normal and functional. The authors suggested that the tissue resistance was not due to a defect in the VDR and that the hormone resistance causing HVDRR was most likely the result of a mutation in an essential protein that participates in the  $1,25(\text{OH})_2\text{D}$  action pathway.

In a follow-up study of this interesting case, Chen et al. [17] proposed that the cause of  $1,25(\text{OH})_2\text{D}_3$  resistance was due to the abnormal expression of a VDRE-interacting hormone response element-binding protein. This protein is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family that binds to double-stranded DNA and modulates RNA processing. This mechanism has led to designating this type of rickets in the category of VDDR-2B along with HVDRR (VDDR-2A) as pathways that block  $1,25(\text{OH})_2\text{D}$  action. The authors proposed that this case of vitamin D resistance in a human subject is similar to that previously described for New World primates in which abnormal expression of a hormone response element-binding proteins can cause target cell resistance to  $1,25(\text{OH})_2\text{D}$  and also to estrogens [17,181]. That this protein is a member of the hnRNP family capable of interacting with double-stranded DNA highlights a potentially important new component of the complex machinery required for steroid hormone signal transduction.

In Cauca, Columbia, more than 200 patients were diagnosed with a disease that somewhat resembles HVDRR without alopecia [182]. The patients exhibit lower limb deformities due to rickets but were otherwise in good physical condition. Rickets limited to the lower extremities, as in these cases, has not been reported in other HVDRR families. The affected individuals had serum calcium levels that were within the normal range but their serum  $1,25(\text{OH})_2\text{D}$  levels were unusually high suggesting target-organ resistance. Fibroblasts from two of the most severely affected patients showed reduced

induction of 24-hydroxylase activity by  $1,25(\text{OH})_2\text{D}$ . However, no mutations were found in the VDR gene. Since the cause of vitamin D resistance in this instance was not due to mutations in the VDR and functional response to  $1,25(\text{OH})_2\text{D}$  was demonstrated, it has not been clearly ascertained whether this entity is a variant of HVDRR or not. The high prevalence of the unique disease in the population and the localized distribution of rickets raise the possibility of an environmental cause. These cases support the concept that targets organ resistance to  $1,25(\text{OH})_2\text{D}$  may be due to mechanisms other than mutations in the VDR or overexpression of components such as an hnRNP that can inhibit  $1,25(\text{OH})_2\text{D}$  signaling.

## 6. Therapy of HVDRR (VDDR-2) patients

### 6.1 Vitamin D

Mutations in the VDR that cause HVDRR reduce calcium and phosphate absorption from the intestine and result in hypocalcemia. The low levels of serum calcium lead to secondary hyperparathyroidism that contributes to the developing hypophosphatemia and to a decrease in bone mineralization that causes rickets. Although endogenous  $1,25(\text{OH})_2\text{D}$  is already elevated, early attempts at therapy focused on the administration of high doses of vitamin D or more active metabolites, the responses have varied widely. From these early studies, it appeared that patients with HVDRR without alopecia responded better to treatment with vitamin D preparations than patients with alopecia [39]. Some patients without alopecia showed improvement both clinically and radiologically after the administration of pharmacological doses of vitamin D ranging from 5000 to 40,000 IU/day [20,21,68]; or 20–200  $\mu\text{g}/\text{day}$  of  $25(\text{OH})\text{D}_3$  or 17–20  $\mu\text{g}/\text{day}$  of  $1,25(\text{OH})_2\text{D}_3$  [21]. One patient (F51) with HVDRR without alopecia with the His305Gln mutation, a contact point for the 25-hydroxyl group of  $1,25(\text{OH})_2\text{D}_3$ , responded to 12.5  $\mu\text{g}/\text{day}$  calcitriol [104,105]. The treatment overcame the low affinity-binding defect and achieved adequate VDR occupancy to mediate normal  $1,25(\text{OH})_2\text{D}_3$  responses. Patient (F4) with the Ile314Ser mutation in the VDR LBD was treated with 1 mg/day of vitamin  $\text{D}_2$  from age 2 to age 18 [68]. At age 20 following an uneventful pregnancy, the patient developed hypocalcemia and was treated successfully with 50  $\mu\text{g}/\text{day}$  of  $25(\text{OH})\text{D}_3$ . On the other hand, the patient (F21) with the Arg274Leu mutation in the VDR LBD, a contact point for the  $1\alpha$ -hydroxyl group of  $1,25(\text{OH})_2\text{D}_3$ , failed to respond to treatment with huge doses of vitamin D or its more active metabolites: 600,000 IU vitamin D; or up to 24  $\mu\text{g}/\text{day}$  of  $1,25(\text{OH})_2\text{D}_3$  (calcitriol); or 12  $\mu\text{g}/\text{day}$   $1\alpha$ -(OH) $\text{D}_3$ . The patient later died of pneumonia [31].

Although HVDRR patients with alopecia appeared to be more resistant to treatment with vitamin D metabolites, some of these patients were treated successfully using vitamin D. Two patients (F6, F10) showed signs of improvement when given vitamin D or  $1\alpha$ -(OH) $D_3$  [77,81] and one patient (F15) responded to 25(OH) $D$  as well as  $1\alpha$ -OHD $_3$  [28]. Both  $1\alpha$ -OHD $_3$  and 1,25(OH) $_2D_3$  were also effective treatments in other cases [63,76,79,91,95] including patients (F26, F19) with the Arg50Gln and Arg73Gln mutations [49,65]. Two siblings (F32), with the Glu152stop mutation, showed no increase in serum calcium during high-dose vitamin D treatment despite raising their circulating 1,25(OH) $_2D$  levels to more than 100 times the mean normal range. However, notwithstanding their low serum calcium concentrations, healing of rickets and suppression of PTH were evident [94]. In one case (F3), where vitamin D and 1,25(OH) $_2D$  therapies failed, the patient responded to oral phosphorous treatment [26]. In many cases when patients fail to respond to 1,25(OH) $_2D$ , intensive calcium therapy is used as described below. Since many cases have limited or no response to vitamin D, many patients are now being initially treated with calcium saving time and effort with vitamin D therapy that often fails.

## 6.2 Calcium

Balsan et al. [183], Weisman et al. [184], and Bliziotis et al. [30] early on reported the most significant development in the treatment of HVDRR at the time. In their early studies, they used long-term IV calcium infusions to successfully treat patients with HVDRR who had failed prior treatments with large doses of vitamin D derivatives and/or oral calcium. This novel therapy bypassed the calcium absorption defect in the intestine caused by the mutant VDR. The patient in the Balsan study was infused with high IV doses of calcium during the nocturnal hours over 9 months. Clinical improvement including relief of bone pain was observed within the first 2 weeks of therapy. Within 7 months, the child gained both weight and height. Eventually, the serum calcium normalized, the secondary hyperparathyroidism was reversed, and rickets ultimately resolved as assessed by X-ray and bone biopsy. However, when the IV infusions were discontinued the hypocalcemia and rickets returned [183].

In the Weisman study, two boys aged six and four (F24, F28) with HVDRR, rickets, alopecia, and growth retardation failed to respond to pharmacologic doses of vitamin D or its active metabolites. The boys were then treated with long-term intracaval infusions of calcium through an implantable catheter. A total of 0.5–0.9 g of elemental calcium was infused daily for

18 months and the serum calcium concentration was maintained at 9–10 mg/dL. Bone pain subsided within 1 week of treatment. Serum phosphorus, PTH, 1,25(OH) $_2D$ , and alkaline phosphatase activity were normalized within 4–9 months. Radiographs of the knees and hands showed healing of rickets with complete resolution after 1 year of treatment. The boys grew 12 and 8 cm per year in height. A transilial bone biopsy obtained from one child prior to treatment revealed severe osteomalacia associated with osteitis fibrosa. A follow-up biopsy examined after 12 months of therapy showed almost complete healing of osteomalacia and normal mineralization. Thus long-term intracaval calcium infusions are an effective mode of therapy for HVDRR patients, and when normal serum calcium and phosphorus concentrations are maintained, healing of rickets and normal growth rate could be achieved even in the absence of a functional VDR [184].

In the Bliziotis et al. study [30] unidirectional intestinal fractional calcium absorption (FCA) was measured with stable calcium isotopes in a patient with HVDRR, before the patient was treated with any calciferol. FCA was reduced at 14% (normal, 20%–70%), and no increase in calcium absorption was seen when serum 1,25(OH) $_2D$  levels were more than 50-fold elevated. The patients's cultured skin fibroblasts contained no detectable 24-hydroxylase activity in response to high doses of 1,25(OH) $_2D$ . High-dose IV calcium infusions and oral phosphorus supplementation for 135 days improved or normalized biochemical parameters and resulted in radiographic healing of the rachitic lesions.

Several other groups reported using IV calcium infusions as a therapy for HVDRR. Hochberg et al. [185] reported that in some cases, after radiological healing of the rickets has been achieved with IV calcium infusions, switching to high-dose oral calcium therapy was effective in maintaining normal serum calcium concentrations.

Much of the following description is based on the experience with the Israeli cohort of HVDRR cases (and one of the co-authors of this chapter, D.T.) [186]. This cohort is composed of a group of 28 patients, most with defined VDR mutations, that have been followed for many years. The experience to be described has been gleaned from patients belonging to four families, each family with a different mutation. The patients currently range from newborn to ~36 years of age. Their phenotypes and genotypes have been previously described [25,187]. One family (F11) has a Tyr295stop nonsense mutation in the LBD that truncates the VDR rendering it devoid of biological function [33,62]. Two families have missense mutations in VDR DBD. In one family (F31), a Gly33Asp mutation was identified near the tip of the first zinc finger [65,93]. In the other family (F49), an Arg80Gln mutation was identified in the base

of the second zinc finger [102]. In the fourth family (F24), the patient's cultured skin fibroblasts failed to bind [ $^3\text{H}$ ] 1,25(OH) $_2$ D, and 1,25(OH) $_2$ D treatment failed to stimulate 24-hydroxylase activity. Molecular analysis of the VDR defect was not performed in this patient [185,188]. Other investigators [130,189], shared their experience that in children beyond infancy presenting with severe rickets and bone deformities, the most reliably effective therapeutic measure is long-term IV calcium infusion to heal the "hungry bones". The infusions usually require establishing a central IV port to allow continuous infusions over prolonged periods. In the hands of the Israeli group, the following plan has proved successful. The initial infusion solution of 100 mg of elemental calcium (calcium gluconogalactogluconate) in 500 mL isotonic sodium chloride is increased gradually to doses of 500–1000 mg of elemental calcium. The infusion is administered continuously, 24 h daily during the first month, and gradually reduced to 12 h daily given overnight. The dosage must be adjusted frequently to avoid severe hypercalciuria i.e., keeping the ratio of urine calcium to urine creatinine less than 3 (Ucal/Ucrea <0.3), and to achieve a serum calcium concentration of 2.1–2.5 mmol/L (8.4–10 mg/dL). Cardiac monitoring is important to prevent bradycardia. Clinical improvement is expected within a week of starting intravenous (IV) therapy, at which time bone pain recedes. Some young patients started to walk for the first time within a week of starting IV therapy. Once the "hungry bones" are corrected, transition to therapy with high-dose oral calcium can be successful and is recommended. The group advises a large oral dose of 5–6 g per meter square body surface of elemental calcium. This dose has been shown to be required for some patients to maintain normal calcium and phosphorus levels, thus preventing rickets and promoting normal linear growth. The detailed use of IV calcium with varying protocols in the pediatric population has also been reported by Malloy et al. [23], Forghani et al. [24], and Ma et al. [117].

Delivery of calcium through a central vein, over an extended period of time started in hospital but continued at home, has been associated with significant morbidity. During the initial week of therapy, cardiac arrhythmias, mostly bradycardia, have been observed. Reduction in the infusion rate has been shown to eliminate the bradycardia. Episodes of septicemia occur frequently and require hospitalization, cessation of calcium therapy, removal of the central catheter, and IV antibiotics [23]. Whether this problem is due to decreased ability of HVDRR patients to fight infection or is a problem inherent with long-term IV infusions, especially in the home setting, is not clear [23]. For example, in a case of an infant (F94) with a Leu227Pro mutation, the child developed repeated IV infections in the

intravenous line used to provide calcium [130]. The infant could not be adequately maintained with oral calcium and the investigators felt it necessary to remove the IV line to avoid septicemia. In this case, the clinicians were able to maintain calcium levels above 8 mg/dL using the intravenous preparation of calcium chloride administered via gastric tube [130]. Another example of avoiding a central port was presented by Abali et al. [150] who reported success with IV calcium administered intermittently via a peripheral route in 4 HVDRR children aged 1.5–9.8 years. They reported no complications such as infection, extravasation, or arrhythmias with peripheral infusion over 1–22 months. The treatment normalized PTH and alkaline phosphatase in all patients, after which, oral Ca of 200–400 mg/kg/day and calcitriol of 0.5  $\mu\text{g/kg/day}$  were sufficient to maintain normal PTH levels.

After IV or another mode of calcium delivery normalizes rickets and metabolic abnormalities, the patients can often be switched to oral calcium. Many patients can be maintained on oral calcium thereafter; however, occasionally some patients fall back to hypocalcemia due to decreased compliance or lack of tolerance to the high doses of calcium. IV calcium therapy could then re-instituted.

Several groups [117,135,185,190] have subsequently found that oral calcium in high doses may be successful as initial therapy or follow-up therapy after IV calcium. Initial oral therapy may allow avoidance of ever requiring IV calcium therapy. The dose required is approximately 5–6 g per meter square body surface of elemental calcium but other studies have had success with lower doses. If successful in restoring normocalcemia and reversing secondary hyperparathyroidism, IV infusions with all of their associated problems may be avoided. On the other hand, if the patient has required IV calcium to replenish calcium deficits, a transition to oral calcium should be attempted. For example, in a study by Sakati et al. [88], a patient (F23) who failed to respond to calciferol received 3–4 g of elemental calcium orally per day and showed clinical improvement during 4 months of therapy. The patient cells were later shown to be totally resistant to 1,25(OH) $_2$ D due to a Gly46Asp mutation in the VDR DBD [87].

### 6.3 Cinacalcet

Another adjunctive therapeutic approach to treating the mineral abnormalities in HVDRR is the addition of Cinacalcet to inhibit the secondary hyperparathyroidism as initially suggested by Srivastava and Alon [191]. Secondary hyperparathyroidism from inadequate calcium absorption in the gut as well as lack of PTH suppression by 1,25(OH) $_2$ D and increased excretion of

phosphate, contributes to the underlying pathophysiology for rachitic changes in HVDRR. Srivastava and Alon used Cinacalcet to treat a child with HVDRR in whom conventional modes of IV calcium therapy had to be discontinued. Cinacalcet therapy together with high-dose oral calcium effectively normalized the metabolic abnormalities and the rachitic bone condition. The authors suggested that the relative ease of administration of the calcimimetic as a once- or twice-daily oral preparation, compared with traditional intravenous calcium administration, should encourage its move to the frontline of combination treatment of the acute phase of HVDRR. Others have since reported on the successful addition of Cinacalcet to their treatment regimen [143,148,153,192]. If successful, the addition of Cinacalcet to high-dose oral calcium may allow avoidance of requiring intensive IV calcium with all of its attendant problems.

#### 6.4 Prenatal diagnosis

In affected families, HVDRR can be diagnosed during pregnancy by chorionic villus sampling (CVS) or amniocentesis. In some cases defects in the unborn child's VDR have been detected using [ $^3\text{H}$ ]1,25(OH) $_2$ D-binding assays and 1,25(OH) $_2$ D-induction of 24-hydroxylase activity in cultured cells obtained from CVS or amniotic fluid [188]. In other cases, a prenatal diagnosis of HVDRR was determined by examining DNA from chorionic villus samples for RFLPs generated by known mutations in the VDR gene [193]. Early diagnosis prompts timely treatment soon after birth before bones become undermineralized and severe rickets develops.

#### 6.5 Future therapy using rationally designed vitamin D analogs

Vitamin D analogs have been proposed as a potential therapy for patients with HVDRR especially those with mutations in the VDR LBD [194–198]. The use of vitamin D analogs is based on the rationale that they bind to the VDR at different amino acid contact points than 1,25(OH) $_2$ D. Using cultured fibroblasts from patients and in vitro transactivation assays, the vitamin D analogs 20-epi-1,25(OH) $_2$ D and 1 $\beta$ -hydroxymethyl-3-epi-16-ene-26 $\alpha$ ,27 $\alpha$ -bishomo-25(OH)D were shown to partially or completely restore the responsiveness to the Arg274Leu and His305Gln mutant VDRs but were less effective in activating the Ph2251Cys mutant [194]. These results suggest that mutations that are involved in ligand binding rather than those that are involved in heterodimerization or coactivator binding are more likely to respond to analogs. Future therapy using

vitamin D analogs could be based on a rationale drug design for individual patients with specific types of VDR mutations. However, we realize that the economic feasibility of this approach may be excessive for any individual patient.

### 7. Hypothesis regarding the mechanism for spontaneous healing: outgrowing the need for substantial calcium supplementation in many HVDRR patients after puberty

#### 7.1 Spontaneous healing

An interesting dilemma regarding HVDRR is that multiple patients have had a spontaneous improvement in their rickets but not their alopecia [37,63,64,128]. Spontaneous healing of rickets usually happens between 7 and 15 years of age and was often associated with the time of puberty. See next Section 7.2 below for a hypothesis to explain this effect at puberty. Sometimes the spontaneous recovery occurs after the patient has undergone a relatively ineffective long-term treatment with vitamin D metabolites and mineral replacement. The healing process arises spontaneously and does not appear to be related to the treatment. In some patients, spontaneous improvement occurred after treatment was discontinued [63]. The patients appear to remain eucalcemic with reduced or without calcium supplements and show no evidence of osteomalacia or rickets. Interestingly, cultured fibroblasts obtained from a skin biopsy of a patient taken after spontaneous healing of rickets had occurred were still resistant to 1,25(OH) $_2$ D [63]. Spontaneous improvement has been observed in patients (F18) [37,64] with the Tyr295stop mutation [66,102] and in patients (F19) [63] with the Arg73Gln mutation [65]. Despite the patient's improvement in their hypocalcemia and rickets, the alopecia remained [37,63,64]. It is not clear from the published studies whether this phenomenon of spontaneous improvement with age also occurs in the genetic rickets patients caused by mutations in the *CYP2R1* and *CYP27B1* (VDDR-1) genes. In these cases, where there are active vitamin D molecules that can treat the disease by bypassing the enzymatic defect, there has been less study of spontaneous improvement, although some anecdotal cases of improvement with age have been noted by investigators who care for these cases [199,200]. See Chapters 66 and 67 for a full discussion of VDDR-1 entities.

#### 7.2 Studies of calcium absorption and puberty

As discussed above, during the time surrounding puberty and into young adulthood, many but not all



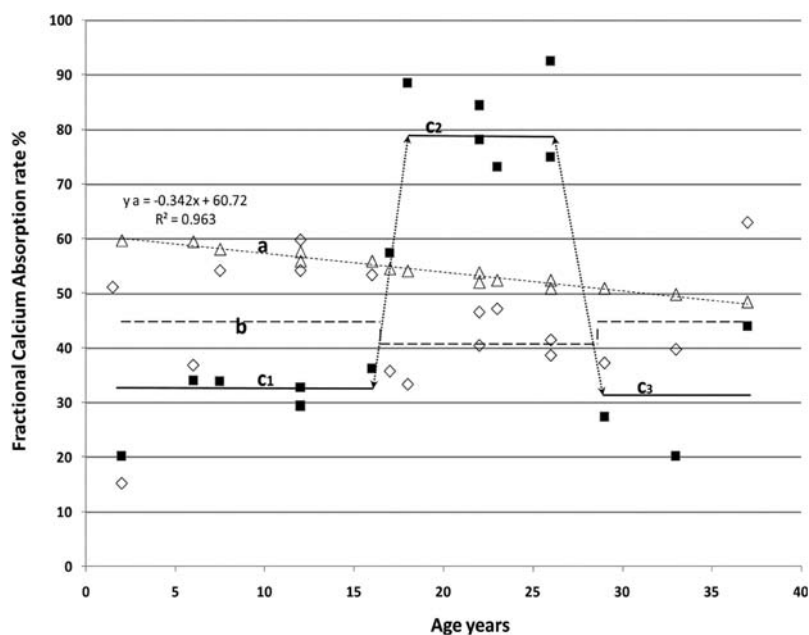
HVDRR patients are able to maintain normal serum calcium levels with more modest oral calcium supplements or, in some cases, even without supplements. Many patient's overt metabolic abnormalities shift toward exhibiting near-normal PTH levels with normal bone mineral density, although they continue to show elevated serum  $1,25(\text{OH})_2\text{D}$  levels, suggesting residual target organ resistance. The alopecia does not improve, again suggesting it is caused by a separate defect, due to mutated unliganded VDR and not vitamin D deficiency or resistance per se. In an attempt to understand the recovery phenomena, Tiosano et al. [201] studied calcium absorption in 17 HVDRR patients 1.5–37 years and age matched controls at different ages and found that they had a unique profile of intestinal calcium absorption and bone mineral accretion (Fig. 68.6). Fractional calcium absorption (FCA) and bone calcium accretion ( $\text{Vo}+$ ) were determined by stable-calcium isotopes. BMD was determined by dual-energy X-ray absorptiometry and bone structure by high-resolution magnetic resonance imaging.

FCA in the HVDRR patients was reduced under a low calcium diet from early childhood until the end of puberty compared to normal age-matched controls. However, from the end of puberty to ~28 years of age and possibly much longer, the HVDRR patients displayed a unique adaptation to low calcium intake, with a significant increase in calcium absorption that reached levels even higher than those of normal controls (Fig. 68.6). Bone calcium accretion was also higher in HVDRR patients compared to normal controls from infancy to adulthood, but not during early puberty [201]. As for

bone mineral density (BMD), although bone calcium accretion was higher in HVDRR patients than in controls, femoral neck BMD was significantly lower in patients aged 12–17 years than in patients older than 18 years, while their lumbar spine BMD was normal. The discrepancy between the high bone calcium accretion and the low femoral neck BMD in the younger group may be explained by their high PTH levels. In contrast, the combined significant increase in FCA accompanied by the decline in PTH levels and the presence of sex hormones after puberty appeared to contribute to the normalization of BMD postpuberty. Further investigation of the bone architecture by high-resolution MRI did not demonstrate any significant differences in cortical bone fraction, trabecular bone fraction, trabecular number, trabecular thickness, or trabecular separation between HVDRR patients and controls [201]. It appears, therefore, that adequate calcium supply and not the presence of functional VDR in human bone is critically important for bone development and mineralization, although impairments of bone quality and subclinical abnormalities not detectable by BMD and MRI studies cannot be excluded in HVDRR patients. This subject is extensively reviewed by Goltzman et al. [202] in mouse VDR knock-down models where they have carefully studied bone biopsies and concluded that calcium cannot entirely substitute for vitamin D in skeletal and mineral homeostasis in mice but that the two agents have discrete and overlapping functions (see Chapter 30 for more discussion).

Previous studies have demonstrated that estrogens regulate a vitamin D-independent pathway

**FIGURE 68.6 Fractional calcium absorption (FCA) in HVDRR patients and normal controls.** Line (a) (open triangles) represent FCA in normal age-matched control subjects at different ages on a low calcium diet. Line (b) (open diamonds) represents FCA in the HVDRR patients during a high calcium diet. Line (c) (black squares) represents FCA in the same HVDRR patients during a low-calcium diet. Line (c1) are children less than 17 years of age, line (c2) postpuberty ages 18 to 29 and line (c3) are HVDRR subjects older than 30 years. FCA in HVDRR patients aged 18–26 years (line c2) was significantly higher during low calcium intake than in patients younger than 17 years (line c1) and in those older than 29 years (line c3). There were no significant and consistent differences in FCA while on a high calcium intake before and after the end of puberty. The figure is used with permission from [201].



controlling calcium absorption, which likely is the mechanism for the improvement in calcium metabolism coincident with puberty [203–206]. Once the affected children pass through puberty, there is no difference between HVDRR in males and females, neither require high doses of calcium. Some can do well with only 0.5 g/day and some don't need any supplementation [186]. This phenomenon may be explained by the observation that in VDRKO mice intestinal calcium transporter genes are upregulated by estrogens through vitamin D and also VDR-independent mechanisms [203–207]. These studies demonstrated that estrogens regulate a vitamin D-independent pathway controlling calcium absorption, in mice and human cells. Furthermore, Nie et al. [206] showed duodenal calcium absorption in ovariectomized mice was significantly decreased, compared with control female mice, which returned to control level after 17 $\beta$ -estradiol replacement treatment. Estrogen regulated the expressions of TRPV6 and PMCA1b in murine and human duodenal mucosae and SCBN cells (human duodenal epithelial cells). The results from SCBN cells showed that 17 $\beta$ -estradiol regulated calcium influx through the effects of estrogen receptor (ER)  $\alpha$  and  $\beta$  on TRPV6 and PMCA1b, respectively [206].

In boys, it appears to be the same mechanism that overcomes the need for supplementation as in girls, a vitamin D independent pathway mediated by estrogens. The levels of testosterone in the blood are 1000-fold higher than the estrogens in boys. High levels of the CYP19 enzyme (aromatase) immunoreactivity were detected within human gastric glands irrespective of age or sex [208]. These results suggest that human parietal cells synthesize estrogens from androgen precursors within gastric mucosa and subsequently secrete them into the portal vein for systemic effects via a gastric vein or perhaps also directly flow into the lumen and down the GI tract.

Once the testosterone level is high enough at puberty in boys, it can be converted to estrogen in the GI tract to enhance the local expression of calcium transporters [208]. Estrogens and vitamin D are thus independent potent regulators of the expression of the calcium influx channels TRPV6 and PMCA1b [206], which are involved in active intestinal calcium absorption. Estrogens and prolactin have also been shown during pregnancy or lactation to have distinct, vitamin D-independent effects at the genomic level on active duodenal calcium absorption mechanisms [207], so multiple factors acting independently of the VDR may be playing a role at different times depending on the physiological situation.

Studies on this hypothesis in HVDRR patients are continuing in order to gain further evidence to support the theory that estrogens are the mechanism for the

phenomenon of HVDRR patients no longer requiring high-dose calcium supplements after puberty. Also, in cases of VDDR-1A and 1B vitamin D deficiency due to synthetic enzyme mutations, there may be some evidence developing that a similar improvement in bone status occurs in adults [199,200]. As seen in Fig. 68.6, in HVDRR patients the FCA rises between age 15 and 30 but seems to fall again after age  $\sim$ 30. This fall in FCA will be the focus of future study. It will be of great interest to follow the status of the calcium levels and bone density in HVDRR adults as they age and the women cross menopause.

## 8. Alopecia

### 8.1 HVDRR (VDDR-2A) and alopecia

The main unresolved medical problem of some HVDRR patients is persistent alopecia despite normalization of both serum biochemistry assays and rickets. The number of hair follicles seems to be normal. However, hair does not grow during childhood and the hair follicles are empty. In some cases papular lesions develop on the face and shoulders, but usually not on the scalp. Both the clinical presentation and the hair histology are similar to those found in the autosomal recessive disease total alopecia known as "atrachia with papular lesions" [209–211] due to mutations in the *hr* gene [212]. The alopecia is socially troublesome and is sometimes made more acceptable using a wig, but normalization of calcium does not improve the alopecia in those that have it.

The mechanism of how the VDR regulates hair growth is still being completely elucidated but efforts from the Demay group and others [213] are providing insight into the mechanism behind the linkage to vitamin D and the VDR which is discussed in Chapter 25. Understanding gained from the molecular analysis of the VDR from HVDRR patients with and without alopecia along with studies in VDR knockout mice (Chapter 30) has revealed a number of interesting findings concerning the role of the VDR and vitamin D in regulating hair growth. First, alopecia is not found in other patients with a genetic deficiency from birth including CYP27B1 mutations (VDDR-1A), CYP2R1 mutations (VDDR-1B), gain of function mutations in CYP3A4 (VDDR-3) or other forms of vitamin D deficiency including severe nutritional deficiency. In the *Cyp27b1* knockout mouse model, abnormalities develop in skeletal, reproductive, and immune function [214]. However, the *Cyp27b1* knockout mice do not develop alopecia. These findings indicate that 1,25(OH) $_2$ D itself is not required for hair development.

Studies of the mutations in HVDRR patients have revealed several interesting findings about the

mechanism of alopecia. HVDRR patients with VDR mutations in the DBD or with premature stop mutations all had alopecia. The alopecia remained unchanged in patients after successful therapy of hypocalcemia and in patients that showed spontaneous improvement in rickets with or without calcium supplementation. Alopecia was also present in patients with mutations that affect RXR $\alpha$ -heterodimerization (i.e., Phe251Cys, Gln259Pro and Arg391Cys). On the other hand, patients with mutations that affect ligand binding (i.e., Arg274Leu, His305Gln, Ile314Ser, and Trp286Arg) did not have alopecia.

Perhaps the most interesting case was a patient (F60) with the Glu420Lys mutation in helix H12 [32]. The Glu420Lys mutation had no effects on ligand binding or RXR heterodimerization but blocked coactivator interactions. However, this patient did not have alopecia and was resistant to even high doses of the hormone. Disruption of coactivator interactions is also implicated in the case of F118 with an Arg343His mutation described above, however, the patient did have alopecia [146]. A second patient with an Arg343Cys mutation also had alopecia [129]. Arg343 is located in helix H9 that is important in RXR heterodimerization but no defect was found in RXR heterodimerization in the Arg343His mutant VDR. Transactivation of the Arg343His mutant VDR was not rescued by the addition of the coactivator GRIP-1 suggesting a defect in coactivator interaction. Modeling shows that the Arg343His mutation blocks the interaction between Arg343 and Gln259 in helix H5 and alters the formation of the cleft needed for coactivator binding [146]. These findings suggest that mutations that affect helix H5 and H12 have differential effects on alopecia even though they both appear to affect coactivator interactions.

Additional evidence supporting these findings include studies in VDR knockout mice that showed that these mice develop alopecia despite calcium supplementation that maintains calcium levels and prevents rickets, indicating that the intact VDR itself (unliganded) is essential for hair growth [215,216]. Also, targeting WT VDR to keratinocytes in *Vdr* knockout mice prevents alopecia [217]. Importantly, when ligand-binding defective or coactivator-binding defective mutant VDRs were specifically expressed in keratinocytes in *Vdr* knockout mice with alopecia, hair growth was fully or partially restored [47]. A role for RXR in hair growth is clearly established as targeted inactivation of RXR $\alpha$  in keratinocytes also causes alopecia [218].

The alopecia associated with HVDRR is clinically and pathologically indistinguishable from the generalized atrichia with papules found in patients with mutations in the *hr* gene [43,44,219,220]. The *hr* gene is expressed in many tissues especially in the skin and brain [221]. The *hr* gene product, HR, acts as a

corepressor and directly interacts with the VDR, and suppresses 1,25(OH) $_2$ D-mediated transactivation [222–224]. Like the VDR, HR is a zinc finger protein suggesting that it interacts with DNA. It has been hypothesized that the role of the VDR in the hair cycle is to repress the expression of a gene(s) in a ligand-independent manner [5,10,32,47,113,222,224]. The ligand-independent activity requires that the VDR heterodimerize with RXR and bind to DNA even if it failed to activate gene transcription [5,32]. The corepressor actions of HR may also be required in order for the unliganded VDR to repress gene transcription during the hair cycle. Mutations in the VDR that disrupt the ability of the unliganded VDR to suppress gene transcription are hypothesized to lead to the derepression of a gene(s) whose product, when expressed inappropriately, disrupts the hair cycle that ultimately leads to alopecia [5,10,47,113,222,224].

RNA-Seq studies by Saini et al. [213] found that >80% of differentially expressed genes were upregulated in keratinocyte stem cells (KSC) from *Vdr* knockout mice demonstrating that the VDR suppresses transcription in WT-KSCs. Among the genes that were upregulated, PPAR $\gamma$ , a major regulator of adipogenesis, emerged as a candidate for further study. The authors showed that the VDR was recruited to the regulatory region of PPAR $\gamma$  gene. Furthermore, they demonstrated that preventing PPAR $\gamma$  overexpression restores hair regrowth. Supporting evidence for the actions of unliganded VDR was presented by Lee and Pike [225] comparing *Vdr* null and *Cyp27b1* null mice concluding that the VDR may function as a selective suppressor/derepressor of gene expression in the absence of 1,25(OH) $_2$ D. See Chapter 25 for further discussion.

## 9. Extra-skeletal actions of vitamin D in HVDRR (VDDR-2A) patients

### 9.1 Extra-skeletal aspects of HVDRR

As mentioned above and discussed extensively in many chapters in this volume, in addition to maintaining calcium homeostasis, 1,25(OH) $_2$ D has been shown to regulate multiple nonskeletal biological processes in many tissues and malignancies including the immune system [226–235]. Expression of *VDR* and *CYP27B1* has been found in many tissues and cancers that have been tested and evidence of functional responses in all these diverse parts of the body including cancer and the immune system are now commonplace [226–235]. The *Vdr* knockout mouse model has been used to analyze the abnormalities caused by the loss of VDR action in detail not possible in HVDRR patients (see Chapter 30).

The clinical findings in a limited number of children and young adults who constitute what we refer to as the Israeli Cohort have been followed medically for years [186] and will be further discussed below in the following sections. Although there are many pleiotropic tissue responses regulated by  $1,25(\text{OH})_2\text{D}$ , children and young adults with HVDRR appear relatively normal except for the constellation of features that relate to their calcium deficiency, rickets, secondary hyper-parathyroidism and alopecia if present. The entire cohort of 26 HVDRR patients from delivery to 46 years of age maintains a normal lifestyle. They exhibit none of the diseases being studied for increased risk or worse prognosis in vitamin D deficiency including cancer, autoimmune disease, cardiovascular disease, diabetes, and others. The married men and women have normal pregnancies and healthy babies delivered at term. They take no medications except some take modest calcium supplements.

## 9.2 The immune system in HVDRR patients

VDRs have been found in hematolymphopoietic cells and  $1,25(\text{OH})_2\text{D}$  has been shown to regulate cell differentiation and the production of interleukins and cytokines [226,235] (see several chapters in Section 11 of this book). Neutrophils isolated from HVDRR patients exhibit only minor aberrations in their fungicidal activity [236], and HVDRR patients have no clinically apparent immunologic defects. Tiosano et al. [237] studied the monocytes of HVDRR patients and found that activating toll-like receptors (TLRs) in the presence of vitamin D initiated a cascade that enhanced *VDR*, *CYP27B1*, and *hnrNP* expression and down-regulated *TLR2* expression.

During the COVID-19 pandemic, among 36 Israeli HVDRR patients, two were found to be positive for SARS-CoV-2 and they had very mild disease. In line with this observation, an HVDRR patient with a DBD mutation and her heterozygotic parents all had mild COVID illness [152,238]. The R80W mutation present in the affected child is located in the C terminal part of the second zinc finger in the DBD of the VDR. Although all family members generated comparable levels of IgG antibodies against the receptor binding domain of the SARS-CoV-2 spike protein as seen in COVID-19 convalescent serum in the general population, their T-cell responses were decreased. Interestingly although the heterozygous family members did not exhibit any signs of HVDRR, their T-cell responses were reduced by 50% commensurate with the percent of wild-type VDR present [152]. Vitamin D and the COVID-19 pandemic are discussed in more detail in Chapter 100.

## 9.3 Autoimmune disease and cancer

Autoimmune diseases, characterized by an inflammatory autoimmune response against self, are the third

leading cause of morbidity in the industrialized world and a leading cause of mortality among women [235]. Autoimmune diseases are chronic conditions with increasing prevalence with age. In a substudy of data from the large VITAL trial that included 25,871 participants, consisting of men  $\geq 50$  years and women  $\geq 55$  years at enrollment, vitamin D supplements were given for 5 years, with or without omega 3 fatty acids. Vitamin D supplementation reduced autoimmune disease by 22%, while omega 3 fatty acid supplementation with or without vitamin D, the reduction was not statistically significant. So although these subjects did not have HVDRR, the importance of the finding was to demonstrate that vitamin D regulates a wide array of genes involved in inflammation and immunity. This study of more than 25,000 older adults in the US provides evidence that daily supplementation with 2000 IU/day of vitamin D or a combination of vitamin D and omega 3 fatty acids for 5 years reduced autoimmune disease incidence. The clinical importance of these findings is high and the results were positive across different autoimmune diseases and demonstrated increasing benefits with time [235]. Whether the HVDRR subjects will show increased problems with autoimmune disease as they age will of course be carefully watched. Vitamin D effects on various autoimmune diseases are discussed in several chapters in Section 11 of this book.

Similar benefits in improving survival in cancer patient were also reported from a reanalysis of the VITAL trial. In the follow-up analysis [239], that accounted for cancer latency by excluding the first year or first 2 years of follow-up, the improvements in survival were statistically significant and as high as 22% and 25% improvement respectively. The authors concluded that updated meta-analyses of randomized controlled trials that include VITAL and other recent vitamin D randomized controlled trials indicate a significant reduction in cancer mortality but not in cancer incidence or cardiovascular disease endpoints [240]. The effects of vitamin D on cancer are discussed in multiple chapters in Section 10 of this book. So the question is whether the HVDRR-affected individuals will lose the protection offered by vitamin D activity protecting against cancer and autoimmune disease as they grow older or will other Vitamin D-VDR independent actions also protect them from these diseases just as they appear to do after puberty for intestinal calcium absorption?

## 9.4 Asthma in HVDRR patients

VDR and  $1\alpha$ -hydroxylase (*CYP27B1*) are expressed in immune system cells as well as in respiratory tract epithelial cells [241,242]. The local extrarenal production of  $1,25(\text{OH})_2\text{D}$  in the presence of functional VDR enables intracrine/paracrine actions of vitamin D in the immune system as well as in respiratory tract epithelial



cells [241]. Bar-Yoseph et al. [243] studied the role of the VDR in lung function, in airways and systemic markers of inflammation, as well as in allergy, among HVDRR patients. Those patients failed to generate airway hyperactivity responses following methacholine challenge test, similar to *Vdr* knockout mice that failed to generate airway hyperactivity responses [244]. The findings suggest that the VDR is required for the generation of cytokines involved in the hyperactivity response and lung inflammation. The exhaled breath condensate cytokine profile in HVDRR patients showed significantly higher IL-4 and IL-17 concentrations compared to controls, while IL-5, IL-10, and interferon- $\gamma$  concentrations were significantly lower. The results obtained from the HVDRR patients suggest that although the pro-inflammatory cytokines IL-4 and IL-17 are elevated, the recruitment of other immunological factors is needed for the escalation of the asthmatic cascade. None of this group of HVDRR subjects has developed asthma. The potential contribution of vitamin D deficiency to the development of asthma is discussed in Chapter 107.

## 9.5 The cardiovascular system in HVDRR patients

Several studies have shown that the VDR in adult rat and mouse cardiomyocytes is located in the t-tubular structure [245]. Ablation of the VDR in mice resulted in chronic changes in contractile kinetics: specifically, 1,25(OH) $_2$ D had rapid effects on myocyte contraction that were absent in *Vdr* knockout myocytes [245]. It was proposed that calcitriol (1,25(OH) $_2$ D) acts directly on the heart as a tranquilizer by blunting cardiomyocyte hypertrophy [246]. Based on those observations, Tiosano et al. [247] studied the renin-angiotensin system, the blood pressure levels, and the cardiac structure of 17 HVDRR patients (9 male and 8 female). All patients had normal blood pressure measurements during ambulatory blood pressure monitoring. Furthermore, none had blunted nocturnal dipping during normal night sleep ("nondipper profile"), which, if dipping is present, is an early and sensitive sign of a general tendency toward hypertension. None of the HVDRR patients had any evidence of increased renin-angiotensin system activity or pathological hypertrophy of the heart, at least prior to the age of 36 years. These findings indicated that from 6 to 36 years of age, humans with HVDRR have normal renin and angiotensin-converting enzyme (ACE) activity, mild but nonsignificant elevation of angiotensin II, normal aldosterone levels, and no hypertension or gross cardiac abnormalities. The effects of vitamin D on the renin-angiotensin system are discussed in detail in Chapter 41, effects on

hypertension and cardiovascular disease are discussed in Chapter 78.

## 9.6 Reproductive history of HVDRR patients

VDR is expressed throughout the organs of reproduction, including oocytes, theca and granulosa cells, endometrium, and placenta in females, whereas in males VDR is found in Leyding cells, germ cells, mature spermatozoa, epididymis, and prostate suggesting a direct role for vitamin D in reproduction (Chapters 39 and 40).

Tiosano and Weisman [248] studied the reproductive history of 16 HVDRR patients (4 married men, 2 married women, 7 single women, and 3 single men). The subjects all had normal calcium and phosphorous levels either with or without modest calcium supplementation after reaching puberty. All of these HVDRR patients had normal pubertal development. The mean age at menarche of HVDRR women was  $13.8 \pm 0.8$  years and they all had normal and regular menstrual cycles. Two married women reported four pregnancies and they have four healthy babies born at term. The time to conceive in the HVDRR women was less than 1 year. Four married HVDRR men reported 15 pregnancies. Nine healthy babies were born. The time to conceive in the wives of the HVDRR men was less than 1 year. Semen analysis obtained from HVDRR men showed normal parameters including viscosity, PH, concentration, total count, motility, total motile count, vitality, and morphology. This study revealed that normocalcemic and normophosphatemic HVDRR patients have a normal reproductive history despite the lack of biologically active VDR. The study suggests that intact VDR is not crucial for reproduction and that the physiological role of normal VDR in reproduction of both men and women remains unclear.

## 10. Conclusions

HVDRR (VDDR-2A) is a rare recessive genetic disorder caused by heterogeneous mutations in the VDR that result in end-organ resistance to 1,25(OH) $_2$ D action. The classical role of 1,25(OH) $_2$ D is to regulate mineral homeostasis, achieved through its coordinated actions on the intestine, kidney, bone, and parathyroid gland. The major manifestation of the defective VDR on the vitamin D endocrine system is to decrease intestinal calcium and phosphate absorption that results in decreased bone mineralization and rickets. Secondary hyperparathyroidism follows as a consequence of the hypocalcemia and loss of feedback inhibition on PTH secretion, problems that cannot be overcome by the elevated levels of circulating 1,25(OH) $_2$ D.

The VDR is also expressed in a wide variety of normal and malignant tissues, including cells of mesenchymal and hematopoietic origin including the immune system [40,41,226–235]. From the ubiquitous nature of the VDR, it appears that the role of the VDR in cellular function is not simply homeostatic but rather pleiotropic. The expanded scope of vitamin D actions includes stimulation of differentiation, inhibition of cell proliferation, and modulation of the immune response in normal and malignant cells [4,227–235]. In addition, the regulation of cellular proliferation and differentiation by  $1,25(\text{OH})_2\text{D}$  is a common feature in many tissues that have been examined, and it is likely that this regulatory feature is a fundamental component of all biological responses to  $1,25(\text{OH})_2\text{D}$ . Notwithstanding the complexity and diversity of biological responses elicited by  $1,25(\text{OH})_2\text{D}$ , the profound skeletal abnormalities demonstrated in patients with HVDRR emphasize the fundamental and essential role of  $1,25(\text{OH})_2\text{D}$  in calcium homeostasis up until puberty, after which other factors subsume partially or completely the ability to regulate intestinal calcium absorption.

It is interesting to note that despite the many pleiotropic processes regulated by  $1,25(\text{OH})_2\text{D}$ , children with HVDRR exhibit only symptoms that relate to their calcium deficiency and alopecia if they have it. After normalization of calcium, they appear normal in most respects except for alopecia, when present, and after puberty, many no longer require calcium supplements. Analysis of HVDRR patients has provided many interesting insights into vitamin D physiology and the role of the VDR in mediating  $1,25(\text{OH})_2\text{D}$  action. However, the potential lack of an impact of loss of normal  $1,25(\text{OH})_2\text{D}$  actions in the young adult HVDRR population on the many diseases described in this book, such as cancer, diabetes, hypertension, autoimmune disease, etc., is as yet not fully understood. It should be kept in mind that the number of HVDRR subjects being followed is very limited, still mostly young, and over time more health problems may become apparent. However, at this time, the relative normalization of the general health of the HVDRR-affected subjects after puberty represents a dilemma that is not yet explainable. All patients followed in this cohort have normal liver and kidney function, none have cancer, diabetes, hypertension, autoimmune disease, endocrine disease, or cardiovascular disease and they do not require treatment with any medication other than that some still require calcium. All of the married individuals have had normal reproduction with healthy babies at term [186,248].

Another somewhat confusing finding in the HVDRR population is that even the problems of reduced calcium absorption and rickets seem to abate after puberty. We have discussed a hypothesis to explain this finding by pointing out that fractional calcium absorption, which

is low in children with HVDRR, rises substantially after puberty. This has led to the hypothesis that the improvement is due to vitamin D and VDR-independent actions to improve calcium absorption and that the mediator of these actions is the rise in estrogens at puberty. Estrogens have been shown earlier to mediate GI calcium absorption by a vitamin D-independent action [203–206]. But boys also show improvement in calcium absorption and here the hypothesis falls back on the presence of aromatase in the human GI tract [207] allowing for the local conversion of androgen precursors to estrogens in males with HVDRR to also improve calcium absorption via estrogenic action in postpuberty males. Thus, these data suggest that a vitamin D and VDR-independent mechanism can ameliorate the clinical difficulty caused by a mutation in the VDR that genetically inactivates vitamin D-dependant pathways.

Of all of the clinical findings in HVDRR-affected individuals, the major one that seems permanent is alopecia, in the affected persons that have it. Alopecia appears to be due to selective defects in the unliganded VDR protein and it is unrelated to the vitamin D molecule and its absence or deficiency. Alopecia is not seen in nutritional vitamin D deficiency or mutational defects in synthesis (VDDR-1s) or accelerated degradation (VDDR-3). Patients with DBD mutations and premature stop mutations in the VDR all have alopecia and are totally hormone-resistant. Based on which mutations cause alopecia and which ones do not, we concluded that mutations that affect DNA binding, VDR-RXR heterodimerization, or that truncate these critical regions of the VDR are all linked to alopecia while mutations that affect only ligand binding or possibly some coactivator binding functions are not. Some selective mutations that affect coactivator binding are also associated with alopecia. This indicates that VDR-RXR heterodimerization and DNA binding are critical for VDR function in hair development. A role for RXR is clearly demonstrated since targeted inactivation of RXR $\alpha$  in keratinocytes also causes alopecia [218].

In conclusion, the biochemical and genetic analysis of the VDR in HVDRR (VDDR-2A) patients has yielded important insights into the structure and function of the VDR in mediating  $1,25(\text{OH})_2\text{D}$  action. Mouse models of HVDRR created by inactivating or deleting the VDR have proved valuable adjuncts to the study of human HVDRR. Studies of the affected children with HVDRR continue to provide further insight into the biological role of  $1,25(\text{OH})_2\text{D}$  in vivo. However, the findings that many young adults with HVDRR, essentially a human VDR knock-out, are functioning normally without apparent abnormality or disease, raises important and difficult questions about extra-skeletal vitamin D actions throughout the body for which there is a growing body of evidence as detailed in many chapters in this book. As

in the example of improved GI calcium absorption after puberty leading to a cure of rickets, alternative vitamin D and VDR independent actions may also play a role in attempting to normalize the biology in the population with an inactive VDR, as such a mechanism mediated by estrogens appears to do in HVDRR affected children after puberty.

A concerted investigative approach of HVDRR at the clinical, cellular, and molecular levels and useful animal models has proven exceedingly valuable in understanding the details of the mechanism of action of 1,25(OH)<sub>2</sub>D and the role of the VDR. The research has also improved the diagnostic and clinical management of this rare genetic disease. But several critical issues remain to be resolved including the role of vitamin D and the VDR, in many common and serious extra-skeletal diseases associated with vitamin D deficiency.

### 11. Summary points

- HVDRR is caused by heterogenous mutations in the VDR.
- The affected children develop severe rickets, hypocalcemia and secondary hyperparathyroidism due to loss of vitamin D action to increase intestinal calcium absorption.
- Therapy is targeted at replacing the deficient calcium with large supplements either intravenous or oral. The use of Cinacalcet to reduce hyperparathyroidism has proven useful as a therapeutic adjunct and appears to reduce the need for intravenous delivery of calcium.
- Restoring calcium to normal with supplements leads to curing of the rickets suggesting that the major benefit of vitamin D action on bone occurs in the GI tract on the calcium absorption pathway.
- Many children exhibit spontaneous improvement of rickets around the time of puberty and some no longer need calcium supplements to maintain adequate calcium levels. The current data indicate the improvement is due to the pubertal increase in estrogens that mediate a vitamin D-independent action to increase intestinal calcium absorption. Males also improve because of aromatase enzyme activity within the GI tract to convert androgens to estrogens.
- A small group of affected children and adults have been followed to determine whether they exhibit an increased risk of developing extra-skeletal diseases like cancer and autoimmune disease that are being studied to determine whether vitamin D deficiency increases the risk and worsens the prognosis. At this time, the small number of subjects and mostly young

adults do not show evidence of increased risk of any disease and appear to have normal reproduction.

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# Infantile hypercalcemia and CYP24A1 mutations

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## OBJECTIVES

- To explain the history and nomenclature for vitamin D–related hypercalcemias.
- To describe the molecular genetics of CYP24A1 mutations.
- To outline the role of CYP24A1 in vitamin D metabolism and biochemical mechanism for CYP24A1-mediated hypercalcemia.
- To delineate the clinical symptoms, diagnosis, and therapy of CYP24A1-mediated infantile hypercalcemia.

## 1. Introduction

Infantile hypercalcemia due to CYP24A1 mutations is reported on the Online Mendelian Inheritance in Man compendium (OMIM, #143880) as a rare condition presenting with failure to thrive, vomiting, dehydration, nephrolithiasis/nephrocalcinosis, and, eventually, death.

Idiopathic infantile hypercalcemia (IIH) is a historical term still used to encompass different conditions characterized by early-onset acute hypercalcemia, often leading to a serious clinical picture. From the first independent descriptions of IIH in 1952 by Fanconi [1] and Lightwood [2], its pathophysiology has been extensively studied, and specific loss-of-function mutations in CYP24A1 [3] and SLC34A1 [4] genes, as well as the Williams-Beuren syndrome [5], have been identified as

possible causes of IIH. As a consequence, since the term IIH is nowadays considered a misnomer [6], the new terms infantile hypercalcemia-1 (HCINF1) caused by CYP24A1 mutations and infantile hypercalcemia-2 (HCINF2) caused by SLC34A1 mutations have been proposed.

The cytochrome-P450 24 subfamily A member 1 (CYP24A1) gene encodes for 24-hydroxylase enzyme responsible for the catabolism of both 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] [7] (reviewed in Chapter 5). CYP24A1 loss-of-function may result in increased serum 1,25(OH)<sub>2</sub>D<sub>3</sub> and calcium concentrations associated with low-to-undetectable parathormone (PTH) [8].

Hypercalcemia is a common condition, with an estimated prevalence of 1/500 patients in the outpatient setting [9]. Hypervitaminosis D as a cause of hypercalcemia is often due to vitamin D intoxication and granulomatous diseases, rather than abnormalities of vitamin D metabolism [8]. During the past 10 years, the increased awareness of IIH has led to an increased number of reports of hypercalcemia due to CYP24A1 mutations, resulting in a large literature mainly consisting of case reports or small case series that bring to light many aspects of the disease not available when initially described.

## 2. Historical notes

Following the first descriptions by Fanconi and Lightwood [1,2], more than 200 cases were reported in Great



Britain over 2 years. Original metabolic studies in IIH patients identified increased intestinal calcium absorption and renal calcium excretion [10], which was attributed to an intrinsic sensitivity to vitamin D [11]. When the British Pediatric Association recommended reduction in the dosage of vitamin D in fortified milk products [12], the incidence of infantile hypercalcemia in the United Kingdom significantly declined [13]. Similar findings were reported in other countries using high vitamin D dosages for rickets' prevention (i.e., German Democratic Republic [14] and Poland [15]), but not in the United States, where lower doses of vitamin D were given during infancy [16], suggesting a dose–effect relationship.

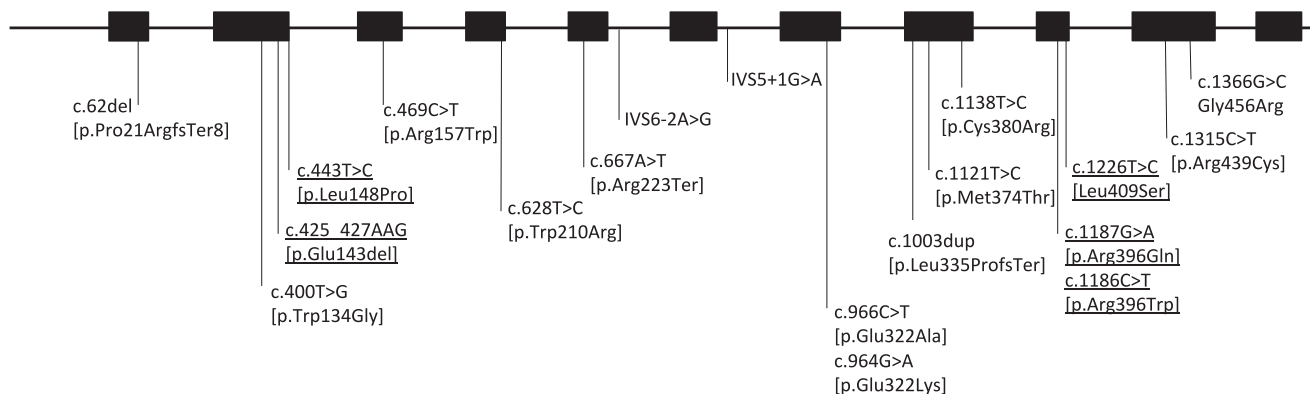
Interestingly, some patients described in the earliest reports presented, in addition to the symptoms of severe hypercalcemia, congenital cardiac abnormalities and an “elfin-like” facies. Accordingly, two different phenotypes were described, namely, “mild” cases, or Lightwood-type IIH, and “severe” cases, or Fanconi-type IIH. Subsequently, when Williams [17] and Beuren [18] independently reported a syndrome (nowadays named Williams–Beuren syndrome) characterized by supravalvular aortic stenosis, peripheral pulmonary artery stenosis, mental retardation, dental abnormalities, and a peculiar facies, it was associated with the Fanconi-type “severe” IIH [19]. Only about 30 years later, the Williams–Beuren syndrome was associated to large deletions on chromosome 7q11.23; this is responsible for the cardiovascular manifestations due to the deletion of the *ELN* gene encoding elastin [20], and, possibly also of vitamin D metabolism impairment due to deletion of other genes on the same chromosome with a mechanism yet to be elucidated [21,22].

At that time, conversely, the pathophysiology of the “mild” Lightwood-type IIH remained unknown, even though the description of family studies suggested its genetic nature [23,24]. Only in 2011, recessive loss-of-function mutations in *CYP24A1* were described in these

patients [3] as the cause of the disease. This mutation was identified using a candidate gene approach, which included *CYP27B1* (1 $\alpha$ -hydroxylase), *CYP24A1*, *FGF23*, and *KL* (klotho). Nonsense or missense loss-of-function biallelic mutations of the *CYP24A1* gene were identified in most patients [15,25–27], including those of the historic cohorts previously diagnosed with IIH [15,27]. A few years later a mutation in the *SLC34A1* gene, encoding the renal sodium phosphate cotransporter 2A, was reported as another possible cause of infantile hypercalcemia in patients resulting without *CYP24A1* mutations [4].

### 3. The genetic basis of the disease: molecular genetics of CYP24A1 mutations

The *CYP24A1* 24-hydroxylase belongs to the cytochrome-P450 superfamily, subfamily A, member 1. This enzyme is encoded by the *CYP24A1* gene, which is located on chromosome 20, position 20q13.2 [28]. This gene is expressed in the whole body, with a particular abundance in kidney, placenta, bladder, and endometrium [29]. Following the discovery that *CYP24A1* mutations were a cause of IIH [3], sequence variations or pathogenic variants in the same gene were identified worldwide [30]. The spectrum of mutations includes missense single-point mutations, nonsense single-point mutations, frameshift mutations (small insertions or deletions leading to a shift in the reading frame of translation), and large deletions of entire exons. These mutations occur all along the *CYP24A1* gene, including the coding exons and in the adjacent splice sites. Fig. 69.1 depicts the distribution of the most frequently encountered pathogenic variants [30]. A recent systematic review and metaanalysis of 50 studies/reports published by the end of 2020 identified biallelic mutations (either homozygous or compound heterozygous) in 61.5% of patients and monoallelic variants in the remaining 38.5% [25]. It is still debated whether the disease



**FIGURE 69.1** Distribution of the pathogenic variants of the *CYP24A1* gene most frequently reported in the literature. The pathogenic variants encountered at least in the 5% of the patients reported before December 31, 2022, are underlined. Modified from Ref. [30].

should be considered as inheritable with an autosomal recessive or dominant pattern. Some authors suggested an autosomal recessive inheritance given the absence of clinical manifestation in patients harboring monoallelic mutations [31,32]. Conversely, other authors, who identified an overt phenotype in these patients, suggested a dominant inheritance pattern [33,34]. Recent studies, which also included subjects wild-type for CYP24A1 mutations, identified an “intermediate” phenotype in patients carrying monoallelic CYP24A1 as compared with biallelic carriers [35], and suggested a “dose–effect” relationship [25,36].

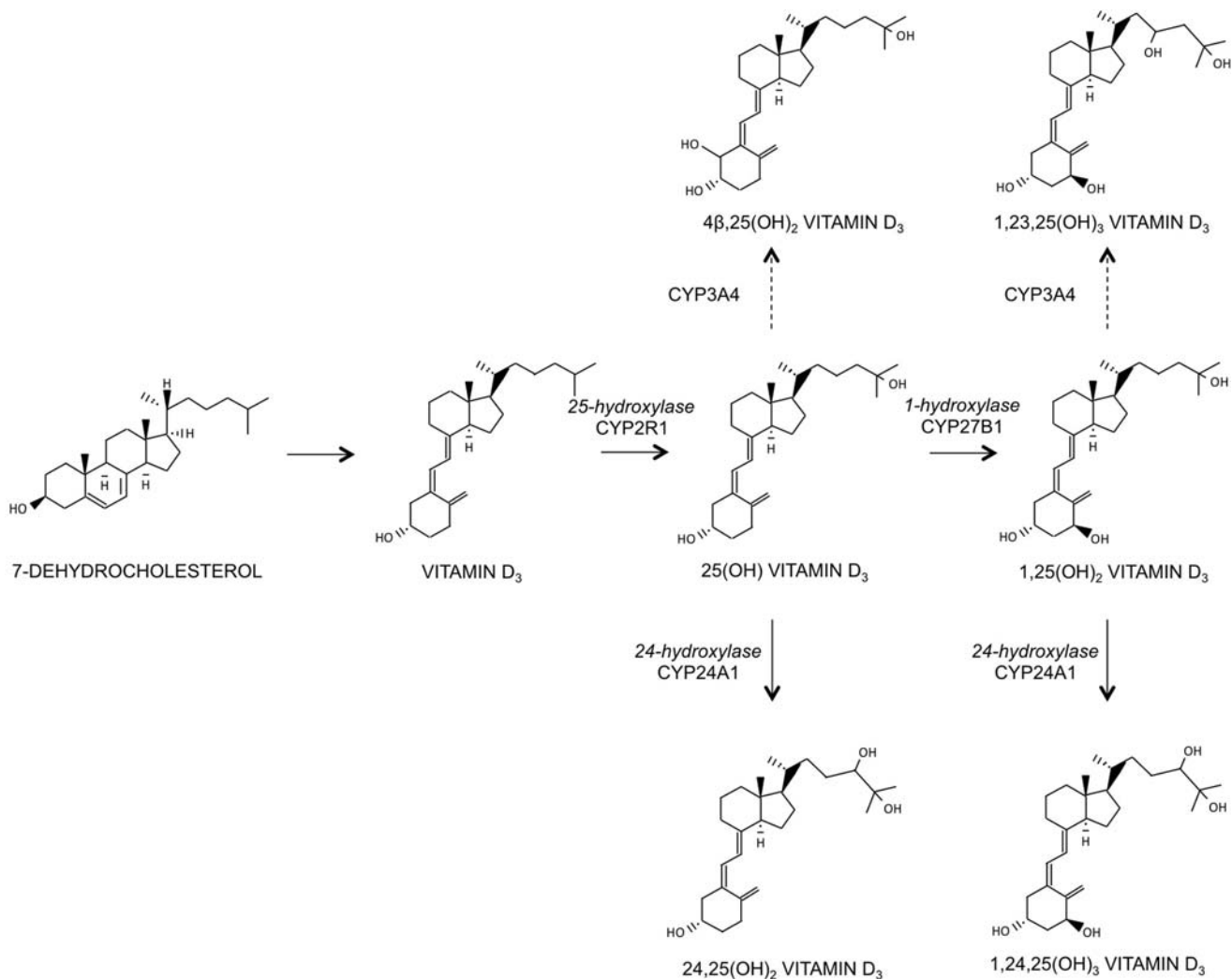
The most common CYP24A1 pathogenic variants are p.Glu143del and p.Arg396Trp, which were associated with infantile hypercalcemia since the first identification of CYP24A1 loss-of-function [3]. These mutations account for about half of the reported pathogenic variants [25]. P.Leu409Ser and p.Leu148Pro are less common (9%

and 5%, respectively), and other variants were mostly anecdotal, often reported once or twice [25].

The prevalence of CYP24A1 mutations in the general population is unknown, since epidemiological data are scarce: an early study reported minor allele frequencies varying from 0.001 to 0.075, with an estimate of total deleterious minor allele frequency of 0.140 [26]. The allelic frequencies for the pathogenic variants estimated by The Genome Aggregation Database (gnomAD) in the aforementioned systematic review and metaanalysis varied from 0.000003977 to 0.008697 [25].

#### 4. The pathophysiology of the disease: mutated CYP24A1 in the vitamin D metabolism

The CYP24A1 gene encodes for a 24-hydroxylase involved in the catabolism of vitamin D (Fig. 69.2)



**FIGURE 69.2** 24-Hydroxylase and vitamin D metabolism. Continuous arrows indicate the physiologic catabolic route, whereas dotted arrows indicate an alternative catabolic route induced by rifampin. Modified from Ref. [30].

(described in more detail in Chapter 5). Vitamin D<sub>3</sub> is hydroxylated in position 25 to 25(OH)D<sub>3</sub>, which is further hydroxylated in position 1 to 1,25(OH)<sub>2</sub>D<sub>3</sub>. CYP24A1 is responsible for the 24-hydroxylation of both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>, which is the first step in their catabolic pathway to 24,25(OH)<sub>2</sub>D<sub>3</sub> and 1,24,25(OH)<sub>3</sub>D<sub>3</sub>, respectively [7,8,28,37]. An impairment in this step leads to an increase in serum 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations. This causes an increase in serum calcium and, to a lower extent, phosphate and a reduction in serum PTH, resulting in a form of vitamin D-dependent hypercalcemia [8].

A CYP24A1 knockout mouse model was generated even before the involvement of this gene in the human infantile hypercalcemia [38,39]. This animal model mimics the phenotype and biochemical parameters of the human disease. These mice have a very high lethality rate during the early infancy and display low baseline 1,25(OH)<sub>2</sub>D<sub>3</sub> levels, a potential compensatory mechanism to prevent vitamin D activation. Both alternative pathways for 1,25(OH)<sub>2</sub>D<sub>3</sub> catabolism by CYP3A4 or a downregulation of 1 $\alpha$ -hydroxylase were suggested [38]. Recently, a knock-in mouse with the R396W mutation of CYP24A1 was generated to further explore the deleterious effects of the most common human mutation as a humanized preclinical model [40].

After the discovery of CYP24A1 mutations, functional studies shed light on the effects of 24-hydroxylase loss-of-function on vitamin D metabolism. Studies using wild-type and mutant CYP24A1 constructs expressed in a mammalian cell line (V79-4, Chinese hamster lung fibroblasts devoid of CYP24A1 activity) confirmed, by measurement of 24-hydroxylated vitamin D degradation products by high performance liquid chromatography, the clinical relevance of the identified mutations [3,32,39].

Additional important information came from the measurement of CYP24A1 activity in vivo by measurement of serum 24-hydroxylated vitamin D metabolites by liquid chromatography/mass spectroscopy (LC-MS/MS) [41]. Several vitamin D metabolites, including 25(OH)D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub>, can be measured, and the 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> can be used as a surrogate parameter for CYP24A1 activity [42].

## 5. Clinical features

In the classical description by Lightwood, hypercalcemia presented in the early infancy, leading to failure to thrive, polyuria/polydipsia, vomiting, dehydration, nephrolithiasis/nephrocalcinosis, and, eventually, death [2].

The age distribution at diagnosis was recently reported to be bimodal, with a first peak in the early

infancy mostly in the first year of life, and a second peak in the adulthood [25]. Most patients with an overt clinical phenotype showed biallelic CYP24A1 mutations, whereas monoallelic variants were often found in relatives of index cases [31,32].

Two phenotypes occur in patients with biallelic mutations: an “early-infancy phenotype” and a “late-infancy/adulthood phenotype.” The former, particularly when presenting in the first year of age [25], is much more severe and characterized by the clinical manifestations of acute hypercalcemia, as originally described by Lightwood. The latter, more often reported in older infants and adults, mostly consists of mild-to-moderate hypercalcemia, recurrent nephrolithiasis (88% of the patients), and nephrocalcinosis (64.7% of the patients) [6,25,32]. The age-related phenotypic switch was early observed and ascribed to the development of tissue resistance to vitamin D, which blunts the hypercalcemic response in the adults but not in the infants [27].

An important issue of the “adulthood phenotype” relates to pregnancy [43–48]. Pregnancy is accompanied by a physiological perturbation to vitamin D homeostasis, especially during the third gestational trimester, when the serum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations increase due to enhanced activity of renal and placental 1 $\alpha$ -hydroxylase [49]. The simultaneous inability to enhance the activity of the placental 24-hydroxylase due to CYP24A1 loss-of-function results in an imbalance between the active vitamin D metabolites and its catabolites [50] (see Chapter 34), which may lead to severe consequences. A recent systematic review reported symptomatic hypercalcemia in the 80% of pregnancies [25], more often presenting during the third trimester, and sometimes even in the postpartum period [43,44]. Clinical pictures of gestational hypercalcemia included neurological (confusion [44], altered mental status [48], and seizures [43,45]) and cardiological manifestations (gestational arterial hypertension, preeclampsia/eclampsia [43,50,51], and rhythm disturbances [43,48]), and two pregnancies complicated by acute pancreatitis [47]. As an outcome, it was reported a 10% rate of spontaneous abortion [25], higher than that observed in the general population according to age and gestational weeks, was reported [52,53]. Therefore, it seems safe to suggest a careful counseling before planning of the pregnancy and an accurate monitoring during the entire pregnancy and the postpartum. Interestingly, female homozygous CYP24A1 (–/–) mice abort their pregnancies [39].

An area of current debate is whether carriers of monoallelic CYP24A1 mutations have a clinically relevant phenotype. With few exceptions [33], monoallelic carriers were reported as clinically unaffected relatives of patients with biallelic CYP24A1 mutations. Interestingly, a recent systematic descriptive review reported that in

this setting, one patient over five presented an overt clinical phenotype, even though patients carrying monoallelic mutations displayed a significantly different biochemical phenotype compared with their biallelic counterparts. In particular, nephrolithiasis was present in the 19.4% and nephrocalcinosis in the 4.9% [25] of cases. Such rates were definitely higher than those reported in the general population [54]. The major limitation in addressing this issue is the lack of case–control studies, analyzing differences between carriers of monoallelic and biallelic *CYP24A1* mutations and wild-type subjects. A recent study within a single family suggests that monoallelic carriers present an “intermediate” phenotype between biallelic carriers and wild-type subjects [35]. One possible explanation is that monoallelic carriers might express an overt clinical phenotype (e.g., symptomatic nephrolithiasis) when exposed to triggering factors, namely a vigorous vitamin D supplementation, sunlight exposure, or pregnancy.

## 6. Diagnosis

The diagnostic approach to *CYP24A1* mutations may be challenging, especially outside the setting of acute hypercalcemia [6]. The diagnostic workup requires a thorough personal and family medical history and a comprehensive laboratory testing. Patients harboring biallelic *CYP24A1* mutations present a PTH-independent hypercalcemia highly variable in its severity, but generally mild outside the settings of early infancy and pregnancy [25]. Hypercalcemia is usually associated with hypercalciuria and normal serum phosphate [25]. Seasonal variations in serum calcium concentrations have been reported [35,55], although this aspect has not been taken into account in most reported patients [25]. Serum 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations displayed a high variability: both analytes are frequently in the normal reference range established by the respective laboratories [25], but are often close to the upper normal ranges established by the National Health and Nutrition Examination Survey [56] and by the Institute of Medicine [57]. Conversely, serum 24,25(OH)<sub>2</sub>D<sub>3</sub> is more often reduced. Nevertheless, since 24,25(OH)<sub>2</sub>D<sub>3</sub> may depend on vitamin D status, the use of the serum 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> ratio is considered a more accurate parameter than serum 24,25(OH)<sub>2</sub>D<sub>3</sub> per se for detecting *CYP24A1* dysfunction [41]. The normal 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> ratio is below 25; ratios above 80 are indicative for *CYP24A1* mutations [41,42,58]. Vitamin D–deficient individuals might exhibit 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> ratio between 20 and 50. A note of caution regards patients with chronic renal failure, who may present low levels of serum 24,25(OH)<sub>2</sub>D<sub>3</sub> concentrations and, therefore, high

25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> ratios. 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> is nowadays recognized as a valuable screening tool for the identification of affected patients, even though it has a negligible value in the identification of monoallelic carriers: as a matter of fact, subjects carrying monoallelic mutations often exhibit 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> values within the normal range [32,33]. These findings are also supported by data obtained in *CYP24A1* knockout mice where pathologic 25(OH)D<sub>3</sub>:24,25(OH)<sub>2</sub>D<sub>3</sub> ratios are found only in homozygous knockout state [59]. Once the diagnosis is suspected, the gold standard for the confirmation is the genetic testing [3].

Hypercalcemia due to *CYP24A1* mutations requires a careful differential diagnosis with other more common forms of hypercalcemia, including primary hyperparathyroidism, familial hypocalciuric hypercalcemia, and other types of PTH-independent hypercalcemia such as paraneoplastic hypercalcemia, granulomatous diseases, and vitamin D intoxication. Table 69.1 shows a schematic representation of the diagnostic workout for the differential diagnosis of hypercalcemia. The diagnostic process may be troublesome for the following reasons: (1) hypercalcemia may be mild outside triggering moments; (2) first-line lab evaluations may provide no clear clues; (3) the “gold standard” screening tool relies on LC-MS/MS, which is not widespread; (4) the genetic analysis may be unavailable.

## 7. Therapy

The management of hypercalcemia due to *CYP24A1* variants should distinguish between an acute approach, aimed to reduce/normalize serum calcium concentration, and a maintenance approach, aimed to the maintenance of serum calcium concentrations stably within the reference range [6,8]. The management of HCINFI in the acute phase should not differ from the management of acute hypercalcemia due to any other cause [6]. Most patients diagnosed in the acute phase should be vigorously hydrated and eventually treated by a loop diuretic such as furosemide when the patient has been hydrated. Additional treatment employed in many reports includes bisphosphonates (pamidronate [43,44,46,60–64], risedronate [63], zoledronate [43]), denosumab [43], azoles (ketoconazole [26,31,34,65–67], fluconazole [68], itraconazole [63]), rifampine [69], glucocorticoids [43–45,61,62,67,68,70,71], calcitonin [44,46–48,62,64], and cinacalcet [63,72].

Each of the aforementioned medications acts on a different step of calcium homeostasis. Azoles reduce the synthesis of 1,25(OH)<sub>2</sub>D<sub>3</sub> by inhibiting the 1 $\alpha$ -hydroxylase enzyme (Fig. 69.2) and are effective in patients affected by *CYP24A1* loss-of-function mutations both in



**TABLE 69.1** Differential diagnosis of hypercalcemic disorders.

Disease	Serum calcium	$Cl_{Ca}/Cl_{Cr}$	PTH	PTHrP	25(OH) D <sub>3</sub>	1,25(OH) <sub>2</sub> D <sub>3</sub>	25(OH) D <sub>3</sub> : 24,25(OH) <sub>2</sub> D <sub>3</sub>	ACE	Genetics
Primary hyperparathyroidism	↑/↑↑	>0.01	↑/inappropriately normal	Undetectable	Variable	↑	Usually <20	Normal	Mutations-specific genes in hereditary forms
Familial hypocalciuric hypercalcemia types 1–3	↑	<0.01	Normal/rarely ↑	Undetectable	Variable	Variable	Usually <20	Normal	Mutations genes <i>CaSR</i> , <i>GNA11</i> or <i>AP2S1</i>
Paraneoplastic hypercalcemia	↑↑↑	>0.01	↓	↑	Variable	Variable	Usually <20	Normal	/
Sarcoidosis/granulomatous diseases	↑	>0.01	↓	Undetectable	Variable	↑↑	Usually <20	Normal/↑	/
Vitamin D intoxication	↑	>0.01	↓	Undetectable	↑↑	↑↑	Usually <20	Normal	/
Hypercalcemia due to <i>CYP24A1</i> mutations	↑/↑↑ (seasonal variations)	>0.01	↓/↓↓ seasonal variations	Undetectable	↑/variable	↑/variable	Usually >90	Normal	Mutations gene <i>CYP24A1</i>

24,25-OH<sub>2</sub> D<sub>3</sub>, 24,25-dihydroxyvitamin D<sub>3</sub>; 1,25-OH<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>; ACE, angiotensin-converting enzyme;  $Cl_{Ca}/Cl_{Cr}$ , calcium clearance:creatinine clearance ratio; PTH, parathormone.

the acute and in the chronic setting [34,68,73]. Rifampin, which activates an alternative catabolic pathway via the CYP3A4 enzyme (Fig. 69.2), thus catalyzing a nonspecific hydroxylation of  $1,25(\text{OH})_2\text{D}_3$  to the inactive metabolite,  $1,23,25(\text{OH})_3\text{D}_3$ , and the 4-hydroxylation of  $25(\text{OH})\text{D}_3$ , is effective in both the short- [69] and the long-term treatment [74]. The use of corticosteroid to reduce intestinal calcium absorption in patients with hypercalcemia related to *CYP24A1* mutations [65] has been discouraged because its therapeutic benefit requires a functioning CYP24A1 enzyme [75]. Nonetheless, corticosteroids have been widely used with good results. One patient underwent hemofiltration [70], and two patients underwent parathyroidectomy [63,72]. The latter patients had coexistence of vitamin D-dependent hypercalcemia and primary hyperparathyroidism: whether this was a causal association or *CYP24A1* mutations are associated with an increased risk of primary hyperparathyroidism remains to be elucidated.

A direct comparison between the efficacy of the various therapeutic strategies in the acute phase is not feasible because available data derived from scattered reports, with a substantial inhomogeneity in the reported hypocalcemic effects, and the lack of randomized controlled trials. Moreover, when considering patients who received a combination of different therapies, it was not possible to understand the actual contribution of each single medication in the overall therapeutic effect.

The chronic management of hypercalcemia in the setting of *CYP24A1* mutations is somehow much more troublesome. Many patients do not require an active pharmacologic treatment [25], and it is reasonable to advise a “behavioral approach”: refrain from exogenous vitamin D supplementation, implementation of a low-calcium diet, and avoid unprotected excessive sunlight exposure. Nonetheless both the strictness and the benefits of these approaches remain to be clarified [37]. For instance, the restriction of dietary calcium should be carefully monitored since it may induce defective bone mineralization and increased intestinal oxalate absorption with the subsequent risk of oxalate stone formation, so there are currently no specific cutoffs for a recommended daily calcium intake. In addition, a recent study has shown that in many cases the behavioral approach alone is not sufficient to induce the normalization of urinary calcium excretion [25,74]. For patients requiring long-term pharmacological management, azoles and rifampin were the medications more often proposed. The efficacy of the different proposed therapies should not only include the hypocalcemic effect both in the acute long-term phases, but also the control of hypercalciuria or the progression of nephrocalcinosis [6,25,74].

An important issue is the management of hypercalcemia during pregnancy, a condition where hypercalcemia may be severe form and most medications cannot be administered [25]. Because of the high chance of obstetric complications, patients should be carefully informed and counseled before planning a pregnancy. Vitamin D supplementation should be avoided since this strategy is as effective in avoiding symptomatic hypercalcemia in repeated pregnancies [71].

## 8. Conclusion

Although frequently undiagnosed, *CYP24A1* mutations can be easily encountered in the clinical practice. The current prevalence of *CYP24A1* pathogenic variants is still unknown, even though it is thought to be high, especially in the European population.

*CYP24A1* loss-of-function mutations are responsible for a highly variable phenotype, ranging from acute hypercalcemia arising in the early infancy with the classical manifestations (nausea, vomiting, dehydration, failure to thrive, up to death), to a much milder phenotype presenting more often in the adulthood with mild hypercalcemia with increased rate of nephrolithiasis. Particular attention should be paid to pregnancy, a condition accompanied by a physiologic modification of vitamin D metabolism, when severe hypercalcemia may occur.

Due to the high phenotypic variability, the possibility of *CYP24A1* mutations should not be suspected only in the limited setting of infantile acute hypercalcemia, but even in each setting of PTH-independent hypercalcemia. Once the diagnosis is suspected, it should be confirmed by studying vitamin D metabolism and by genetic analysis.

The medical approach depends on the entity of hypercalcemia, ranging from vigorous measures aimed at a prompt normalization of serum calcium concentrations, to behavioral measures and lifestyle modifications aimed at a chronic maintenance of normocalcemia.

## 9. Summary points

- Loss-of-function *CYP24A1* mutations are responsible for an increase of  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  concentrations, thus resulting in a form of vitamin D-dependent hypercalcemia.
- The clinical phenotype of patients with biallelic loss-of-function *CYP24A1* mutations varies from severe acute hypercalcemia, more often presenting in the early infancy, to mild hypercalcemia presenting in the adulthood.

- Carriers of *CYP24A1* monoallelic mutations often present with serum calcium concentrations in the upper part of the normal range and have an increased rate of nephrolithiasis.
- The available therapies include first-line measures for the management of acute hypercalcemia, as well as pharmacologic approaches that can be considered, if necessary, for the long-term control of hypercalcemia.

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# Vitamin D and osteoporosis

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## OBJECTIVES

- Explore the effects of season on bone and mineral metabolism, and the evidence for identifying an optimal serum 25(OH)D concentrations for musculoskeletal health.
- Review the evidence for effects of vitamin D alone or calcium and vitamin D on bone mineral density (BMD) and falls.
- Review the evidence for primary fracture prevention with vitamin D or calcium and vitamin D, including metaanalyses and studies of high annual or monthly doses, and to make clinical recommendations based on this evidence.
- Review data on safety relating to calcium and vitamin D, and the evidence for secondary fracture prevention with vitamin D or calcium and vitamin D.
- Review the evidence on the effects of active vitamin D analogs on fractures.
- Review recent evidence on large randomized controlled trials of vitamin D supplementation.
- Review the evidence of vitamin D on bone health from Mendelian randomization studies.

## 1. Introduction

There are powerful inherited contributions to osteoporosis with up to 75% of variance in bone phenotypes, such as bone mineral density (BMD), bone size, and geometry, attributable to genetic factors in

healthy individuals. Allelic variation in the vitamin D receptor (VDR) was the first nonstructural gene to be associated with osteoporosis. These data, in conjunction with the effects of the vitamin D system in bone homeostasis, suggested that this vitamin might have a strong role in osteoporosis treatment. This possibility is consistent with effects of the active vitamin D metabolites directly on osteoblasts to increase bone formation and mineralization and to reduce osteoclast recruitment and increase their activity, while at the same time acting on the osteoclast pathway to increase osteoclast recruitment. In large-scale epidemiological data, serum 25-hydroxyvitamin D (25(OH)D) levels are associated with bone density in both men and women. However, there is mixed evidence on the effectiveness of vitamin D supplementation for the prevention of bone loss and minimal trauma fractures in postmenopausal women and older men. This may be explained by vitamin D being a threshold nutrient, with a threshold in vitamin D status below which disease risk increases and vitamin supplementation is beneficial. In this regard, two recent randomized controlled trials (RCTs) have shown vitamin D supplementation increases bone mineral density in individuals with baseline serum 25(OH)D concentrations <30 nmol/L. There is also likely to be some benefit on primary fracture prevention for those with the highest background fracture risk including those patients who have deficient serum concentrations of 25(OH)D and institutionalized patients, but only when combined with calcium supplements. In women and men aged >50 years, the combination of vitamin D with calcium, but not vitamin D alone, has a modest effect in preventing fractures by 5%–30%, depending on fracture site and study. There is no evidence that vitamin D alone can

prevent fractures in community-dwelling, vitamin D–replete older adults, nor that the combination of calcium and vitamin D, or either alone, can prevent fractures in patients with preexisting minimal trauma fractures. The daily dose of vitamin D should be at least 800 IU (20 µg). There is evidence that daily doses of  $\geq 4000$  IU may be harmful for bone in postmenopausal women, but not in men. Larger monthly or annual doses are also not recommended as in two studies, large single either annual (500,000 IU) or monthly (60,000 IU) doses of vitamin D increased falls and, possibly, fractures in elderly men and women. Vitamin D, calcium, or the combination of both, are not effective in secondary fracture prevention. However, vitamin D supplementation is required in patients with suboptimal serum 25(OH)D concentrations ( $<20$  ng/mL,  $<50$  nmol/L) before antiosteoporosis treatment with bisphosphonates or other antiresorptive drugs, particularly intravenous zoledronic acid and subcutaneous denosumab, to help minimize the risk of hypocalcemia and to optimize the antifracture efficacy of antiresorptive drugs. Active vitamin D metabolites are not recommended for the treatment of osteoporosis.

## 2. Effects of vitamin D on the skeleton

Vitamin D deficiency states result in the failure of normal mineralization of bone. This presents in childhood as rickets and in the mature skeleton as osteomalacia (see Chapters 62 and 63). The consistent finding between these two states is inadequate mineralization of the formed matrix, such that the bones are softened and deformed. There has been considerable discussion as to whether this is entirely due to the major role of active vitamin D metabolites in stimulating gut calcium (and phosphorus) absorption or whether there are additional specific effects on bone. In the former concept, it is proposed that there is failure of the provision of adequate calcium and phosphorus that is required for mineralization to proceed. In the latter, it is proposed that there are additional roles of active vitamin D metabolites in regulation of both bone formation and bone resorption (see Chapters 21–23).

Parathyroid hormone (PTH), which rises in response to low serum calcium, stimulates renal production of 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) that in turn stimulates gut calcium absorption. When there is active absorption of intestinal calcium into the circulation, the rise in serum calcium decreases renal  $1\alpha$ -hydroxylase (the *CYP27B1* gene product) activity both directly and indirectly, through decreasing PTH and increasing fibroblast growth factor-23 levels (see Chapters 8 and 19). This classic negative feedback loop, which has been

well characterized, does not, however, address the direct effect of vitamin D on the skeleton.

The presence of the VDR in bone cells suggests direct effects on bone (see Chapter 23). These receptors are expressed in osteoblasts (see Chapter 22) and in immature cells of the osteoclast precursor lineage (see Chapter 21). It was proposed some time ago that the effect of the active vitamin D metabolites on osteoclasts was indirect via osteoblasts. The clearest potential to discriminate between these direct and indirect vitamin D effects on bone came through the VDR-knockout models [1–8]. In these, there was good evidence that the osteomalacic phenotype could be largely rescued by a high-calcium, high-lactose diet, which overcame the poor gut calcium absorption defect. However, these findings do not preclude other roles of vitamin D in modulating bone cell function. In these studies, a specific role is supported for the VDR in chondrocytes [9,10].

One set of findings that suggest a specific role of vitamin D in bone cells was the overexpression of the VDR in mature cells of osteoblastic lineage [11,12]. These studies reported increased bone mass in mice and stronger bones characterized by increased calcium content of the mineral phase. However, in other studies, VDR knockout was associated with increased osteogenic activity [7]. The diametric difference between these studies is yet to be determined. In relation to the specific VDR overexpression model, there were both in vivo and in vitro studies, demonstrating decreased osteoclastic activity. In the in vitro model, this effect appeared to be driven by the osteoblasts with higher intrinsic VDR levels [13].

Other preclinical models have explored the effects of vitamin D on osteoclastogenesis. Bone volume was positively associated with circulating 25(OH)D concentrations, while osteoclast surface levels were positively associated with receptor activator of nuclear factor- $\kappa$ B ligand (RANKL):osteoprotegerin (OPG) messenger RNA (mRNA) ratio, which were, in turn, higher in groups with lower serum 25(OH)D concentrations, but independent of serum  $1,25(\text{OH})_2\text{D}$  concentrations. Serum 25(OH)D concentrations  $<32$  ng/mL (or 80 nmol/L) resulted in osteopenia due to increased osteoclastogenesis, consistent with human data [14].

The skeleton also appears to be responsive to serum levels of the  $1,25(\text{OH})_2\text{D}$  precursor, 25(OH)D, in terms of bone mineralization [15]. Human osteoclast cultures incubated with 25(OH)D produce measurable quantities of  $1,25(\text{OH})_2\text{D}$ . The expression of osteoclast transcription factor, osteoclast marker genes, and an osteoblast coupling factor, ephrin-b2, is also increased in the presence of 25(OH)D in vitro. Levels of *CYP27B1* and nuclear factor of activated T cells-1 mRNA correlated during osteoclastogenesis and in a cohort of human bone samples. 25(OH)D dependently reduced the resorptive

capacity of osteoclasts, whereas, conversely, osteoclasts formed from VDR-null mouse splenocytes have increased resorptive activity. Thus, it is likely that 25(OH)D metabolism is an important intrinsic mechanism for optimizing osteoclast differentiation and reducing osteoclast activity.

In rodents, the major determinants of serum 25(OH)D are dietary vitamin D and calcium. Compared with animals fed a diet deficient in calcium, animals fed 0.1% calcium had higher renal *CYP27B1* mRNA expression and 12- to 18-fold increased levels of serum 1,25(OH)<sub>2</sub>D [16]. Thus, the reported effects of low-calcium diets on bone loss may be, in part, due to the adverse effects on 25(OH)D metabolism, leading to a reduction in active vitamin D. This potential interaction between dietary calcium and vitamin D metabolism has important implications, given the recent evidence for local synthesis of active vitamin D in bone tissue, and may explain why the combination of calcium and vitamin D is more effective than vitamin D alone in preventing fractures.

In this regard, in the mouse model overexpressing the VDR in both osteoblasts and osteocytes (OSVDR), there was inhibition of bone resorption and increased bone formation. When OSVDR mice were fed a vitamin D deficiency diet, they displayed an increased cortical and cancellous bone volume compared with wild-type mice, which remained higher during vitamin D depletion due to reduced osteoclast numbers and an increased bone formation rate [17]. These data suggest that increased VDR-mediated activity in osteoblasts and osteocytes may prevent bone loss due to vitamin D deficiency.

Overall, these findings suggest two distinct levels of function of the active vitamin D metabolites on bone: a general effect through changes in gut calcium absorption and a fine-tuning effect on the specific activity of the osteoblasts and osteoclasts in bone.

### 3. The role of vitamin D genetic factors in osteoporosis and possible interactions with vitamin D therapy

A variety of risk prediction models have examined family history and shown that a positive family history contributes to risk. Typically, this increased risk has odds ratios of 1.1–1.5. Family history of hip fracture is used in the FRAX fracture risk calculator. The recognition of heritability in osteoporosis led initially to studies of many candidate genes and more recently to large-scale, genome-wide studies. The initial demonstration of the relationship of the VDR to bone turnover and bone density [18,19] was followed by many candidate gene approaches [20–28]. Replication of these findings has been “variable” in different ethnicity and age

samples, even for the two most studied loci, the VDR and collagen Iα1 genes, which are possibly influenced by gene–environment interactions [22,24,28–34]. The gene discovery approach of genome-wide analysis of extreme phenotypes (mutations) in small families led to discovery of the low-density lipoprotein receptor–related protein-5 [35,36]. This work opened understanding of new and previously unsuspected bone anabolic pathways [37–40].

Large-scale, population-based, genome-wide association studies in osteoporosis and fracture risk [41–43] have identified not only, mainly, known bone-related genes but also some other potential genetic targets. The OPG, RANK and RANKL, sclerostin, and estrogen receptor genes were among those to be detected, but several other novel loci are yet to be investigated [42–44]. Another approach has been to perform genome-wide scans in subsamples of high- and low-bone-density individuals selected from normal populations [45].

The primary phenotype in most such studies has been lumbar spine and proximal femur BMD by dual-energy X-ray absorptiometry. This has been extended to a variety of geometric and mechanical parameters, including estimated femoral neck volumetric BMD, width, cross-sectional area, endosteal diameter, mean cortical thickness, cross-sectional moment of inertia, buckling ratio, and section modulus [46–50]. Rate of change of BMD and even fracture incidence have also been considered as phenotypes. Some studies have also reported a strong association between serum 25(OH)D levels and polymorphisms in the vitamin D-binding protein (DBP) gene as well as genes involved in vitamin D metabolism, particularly one of the 25-hydroxylase genes [51–53].

One of the major challenges has been the relatively limited reproducibility of associations between studies. However, this may relate to environmental differences and to ethnic differences in other gene variants. This possibility is supported by recent studies reporting interactions between the variants of the VDR, DBP, and calcium-sensing receptor genes in determining response to therapy including to vitamin D [54–56]. One study has provided new genetic data that might explain why there is considerable interindividual variability of serum 25(OH)D levels [57]. As only 25% of this variability is accounted for by season, latitude, or vitamin D intake, behavioral and genetic factors have been thought to contribute to this variability.

A large, multicenter, genome-wide association study of 15 cohorts in Europe, Canada, and the United States, comprising about 30,000 white people from European descent, found that polymorphisms at three different loci involved in vitamin D metabolism affect serum 25(OH)D levels and the risk of vitamin D deficiency and insufficiency. After accounting for age, sex, body



mass index, and season, polymorphisms in at least three, and perhaps four, loci influenced serum 25(OH)D levels. The first three genes below were identified in the discovery sample and confirmed in the replication sample, while the fourth candidate gene was identified in pooled analyses of the discovery and replication samples: (1) 4p12 polymorphisms near or within the *GC* gene, which encodes DBP, the main transporter of vitamin D metabolites in the blood; (2) 11q12 polymorphisms near *DHCR7/NADSYN1*, encoding the enzyme 7-dehydrocholesterol (7DHC) reductase, which converts 7DHC into cholesterol in the skin, thereby removing the substrate for production of vitamin D<sub>3</sub>; (3) 11p15 polymorphisms near *CYP2R1*, encoding an enzyme responsible for 25-hydroxylation of vitamin D in the liver; (4) *CYP24A1* encoding 24-hydroxylase, which initiates degradation of 25(OH)D and 1,25(OH)<sub>2</sub>D. Participants with a genotype score (combining the three main variants) in the top quartile had twice the risk of having vitamin D insufficiency (<50 or 75 nmol/L) than those in the lowest quartile. It was also associated with a 1.5-fold risk of severe vitamin D deficiency (<20 nmol/L). These findings are important, as they confirm that common genetic variants may contribute to the interindividual variability of serum 25(OH)D levels and may predispose to (or protect against) vitamin D deficiency and insufficiency. This study also raised new questions regarding genetic regulation of serum 25(OH)D: (1) Will the same genetic variants be found in other ethnic groups? (2) Will these genetic variants explain the high variability that is clinically apparent in response to either ultraviolet B (UVB) exposure or to vitamin D supplementation? (3) Are there other less common genetic variants that also affect serum 25(OH)D levels (see Section 12)?

In addition to the role of genetic variants in determining serum 25(OH)D, *VDR* genotypes have been included in a genetic risk score (GRS) for fracture based on 62 BMD-associated single-nucleotide polymorphisms [58]. Individuals with greater GRS had lower femoral neck BMD. In addition, each unit increase in GRS was associated with a hazard ratio (HR) of 1.20 for fracture, independent of age, prior fracture, and falls. This significant association between GRS and fracture was observed for vertebral and wrist fractures, but not for hip fracture. Thus, genetic profiling of BMD-associated genetic variants could improve the accuracy of fracture prediction.

#### 4. Seasonal variation in vitamin D status

Many studies have demonstrated seasonal variations in serum 25(OH)D with a decline during the winter months [59–65]. More importantly, studies from both

the northern and southern hemispheres have shown seasonal variations in serum 25(OH)D concentrations, accompanied by responsive changes in serum PTH concentrations, later increases in bone resorption markers and bone formation markers and decreases in BMD. In 43 German subjects followed for 1 year, bone turnover was significantly accelerated, and lumbar spine and femoral BMD declined by 0.3%–0.9% during the winter months. Supplementation with oral 500 mg calcium and 500 IU cholecalciferol per day for 1 year either reversed or abolished seasonal changes in calciotropic hormones and markers of bone turnover in the intervention group, while these changes remained in a control group. In the subjects receiving oral cholecalciferol and calcium, lumbar and femoral BMD increased significantly, whereas controls continued to lose bone [66].

Rates of hip fracture also vary annually, with a winter peak in both northern and southern hemispheres [67,68]. Inadequate vitamin D levels have been demonstrated in patients with osteoporosis [69], including hip fracture patients in many countries, although low levels may be influenced by the fracture itself [70–72]. It is also important to note that vitamin D deficiency increases with aging and that this contributes independently to secondary hyperparathyroidism [73].

In addition, serum androgen levels in men are associated with serum 25(OH)D concentrations, and both have a concordant annual periodicity being lowest in late winter [74], suggesting important, and as yet unidentified, links between the vitamin D and sex steroid axes. Similarly, in southeastern Australia (latitude 38–39°S), in 287 women drawn from an observational, cross-sectional, population-based study [75], annual periodicities of ultraviolet radiation, serum 25(OH)D, serum PTH, a bone resorption marker, serum C-telopeptide (CTx), BMD, falls, and fractures were measured. Cyclic variations in serum 25(OH)D lagged 1 month behind ultraviolet radiation, peaking in summer and dipping in winter. The periodicity of serum PTH was the inverse of serum 25(OH)D, with a phase shift delay of 1 month. Peak serum CTx lagged peak serum PTH by 1–2 months. In late winter, a greater proportion of falls resulted in fracture. Seasonal periodicity in 439 hip and 307 wrist fractures also followed a simple harmonic model, peaking 1.5–3 months after the trough in 25(OH)D. Thus, a fall in serum 25(OH)D in winter is accompanied by increases in serum PTH levels, bone resorption, and the proportion of falls resulting in fracture, and more hip and wrist fractures [75].

Only one English study did not show such a seasonal variation in bone turnover markers [76]. Season appears to be more important than latitude in determining serum 25(OH)D in many countries. However, in Australia, both season and latitude accounted for less than 20% of the variation in serum 25(OH)D levels,

highlighting the importance of behavioral and genetic factors, including sun avoidance [77]. Such seasonal declines in vitamin D metabolites and the associated increases in hip and wrist fracture rates make interventions with vitamin D a logical intervention.

### 5. Determining optimal serum 25-hydroxyvitamin D concentrations for musculoskeletal health

Previous attempts to define an optimal serum 25(OH)D for musculoskeletal health have used indirect measures such as the relationships between serum 25(OH)D and PTH concentrations in normal adult populations with a plateau of serum PTH above 31 ng/mL (78 nmol/L) [78]. A major role for active vitamin D metabolites is in stimulating gut calcium (and phosphorus) absorption. This effect may not be uniform across all age groups, and in humans, there is evidence of an age-related intestinal resistance to 1,25(OH)<sub>2</sub>D that may be secondary to reduced levels of intestinal VDR [79]. Despite an age-related increase in serum PTH and in serum 1,25(OH)<sub>2</sub>D concentration, intestinal VDR concentration decreased with age, whereas active calcium absorption did not change. These data are most consistent with impaired intestinal responsiveness to 1,25(OH)<sub>2</sub>D action. This gut defect could lead to compensatory increases in PTH secretion and 1,25(OH)<sub>2</sub>D<sub>3</sub> production, which maintain calcium absorption and serum calcium, but at the expense of increased bone loss. Serum 25(OH)D concentrations are also related to active calcium absorption [80], albeit not as strongly, and one study showed a plateau in active intestinal calcium absorption at serum 25(OH)D concentrations  $\geq 32$  ng/mL (80 nmol/L).

A randomized, double-blind, placebo-controlled clinical trial studied 230 postmenopausal women aged  $\leq 75$  years with baseline 25(OH)D levels of 35–68 nmol/mL and no osteoporosis, who were given placebo, 800 IU vitamin D<sub>3</sub> daily, or 50,000 IU vitamin D<sub>3</sub> every 2 weeks for 1 year. The high-dose vitamin D achieved and maintained 25(OH)D levels  $>75$  nmol/L and resulted in small (1%) increases in active calcium absorption. However, this effect did not translate into beneficial effects on BMD, muscle function, muscle mass, or falls [81].

Others have related serum 25(OH)D concentrations to fracture risk. In a nested, case-control study from the Women's Health Initiative, 400 hip fracture patients and 400 controls matched based on age, race, or ethnicity, and date of blood draw were followed for a median of 7.1 years to assess fractures. Lower serum 25(OH)D concentrations were associated with increased hip fracture risk (adjusted odds ratio [OR] for each

25 nmol/L decrease, 1.33). Women with the lowest 25(OH)D concentrations ( $\leq 47.5$  nmol/L) had a higher hip fracture risk than did those with the highest concentrations ( $\geq 70.7$  nmol/L) (adjusted OR, 1.71), and the risk increased statistically significantly across quartiles of serum 25(OH)D concentrations. Importantly, this association was, in part, mediated by increased bone resorption. Thus, serum 25(OH)D concentrations  $\leq 20$  ng/mL (50 nmol/L) are associated with a higher risk for hip fracture [82].

In another study of 1311 community-dwelling older Dutch men and women followed for 6 years, a low serum 25(OH)D level ( $<12$  ng/mL or 30 nmol/L) increased the risk of fracture in those individuals aged 65–75 years (HR = 3.1; 95% confidence interval (CI), 1.4–6.9), but not in the older group of individuals (75–89 years) [83].

The aforementioned inferences about optimal serum 25(OH)D levels are drawn from association studies. Stronger evidence relates to the effects of vitamin D supplementation on musculoskeletal endpoints, particularly in increases in BMD, a reduction in falls and fractures and the serum 25(OH)D threshold concentrations needed to achieve these outcomes. The antifracture efficacy of oral vitamin D supplementation in women and men  $\geq 65$  years old has been assessed in a metaanalysis of 12 double-blind randomized controlled trials (RCTs) for nonvertebral fractures ( $n = 42,279$ ) and 8 RCTs for hip fractures ( $n = 40,886$ ) [84]. The pooled relative risks (RRs) were 0.86 (95% CI, 0.77–0.96) and 0.91 (95% CI, 0.78–1.05) for the prevention of nonvertebral fractures or hip fractures, respectively. However, there was significant heterogeneity for both endpoints. Factors explaining the heterogeneity were the daily dose and achieved serum 25(OH)D concentrations. When examining the trials with a higher (482–770 IU/day) received dose (i.e., dose adjusted for adherence) of vitamin D, nonvertebral fractures were reduced by 20% and hip fractures by 18%, whereas doses  $<400$  IU/day did not show any effect.

Furthermore, vitamin D has been shown to improve muscle strength and performance and to reduce the risk of falling in community dwelling, as well as in the institutionalized elderly. A metaanalysis including eight double-blind RCTs ( $n = 2376$ ) demonstrated that falling was significantly reduced by 13% in vitamin D-supplemented individuals compared with those receiving either calcium or placebo. Again, a significant heterogeneity by daily dose and achieved 25(OH)D levels was observed. Higher doses of vitamin D supplements (700–1000 IU/day) reduced the relative risk of falls by 19% [85,86]. Collectively, this translates to an achieved serum 25(OH)D level of at least 24 ng/mL (60 nmol/L) for antifall efficacy and at least 30 ng/mL (or 75 nmol/L) for antifracture efficacy [87,88].

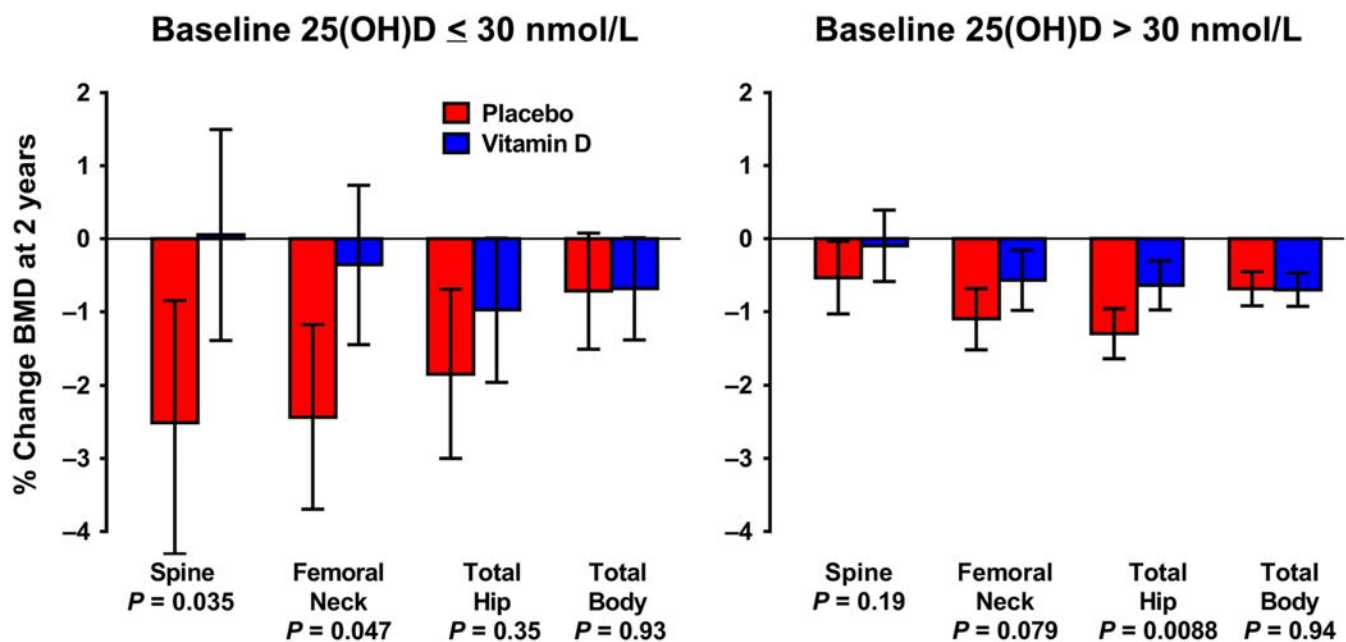
Thus, most clinicians would regard a target range for serum 25(OH)D between 20 and 30 ng/mL (50–75 nmol/L) as being required for optimal musculoskeletal health. However, an Institute of Medicine report recommended at least 20 ng/mL (50 nmol/L) [89], which does not take season into account. Australian guidelines recognize the importance of season and recommend a level of at least 20 ng/mL (50 nmol/L) at the end of winter and early spring [90].

## 6. Effects of vitamin D alone or calcium and vitamin D on bone mineral density and falls

A systematic review of 17 RCTs evaluated the effect of supplemental vitamin D<sub>2</sub> or D<sub>3</sub> on BMD, predominantly in populations of late menopausal women [91]. Most trials had small sample sizes, were 2–3 years in duration, and used vitamin D doses of ≤800 IU per day. In addition, most trials used vitamin D<sub>3</sub> and also included calcium ≥500 mg as a cointervention. Combined results of trials of vitamin D<sub>3</sub> plus calcium versus placebo were consistent with small positive effects on lumbar spine, femoral neck, and total body BMD. The Women's Health Initiative trial found a significant benefit of vitamin D<sub>3</sub> 400 IU plus 1000 mg of calcium on total hip BMD [92]. Although there were no effects on spine BMD, this large study had several potential cofounders including 30% of participants already taking a calcium supplement, 30% having a high dietary calcium intake (>1200 mg/day), 52% receiving current hormone replacement therapy

(HRT), and a large proportion of patients taking a bisphosphonate at study end, as well as poor adherence with calcium and vitamin D supplements.

However, in combined trials of vitamin D<sub>3</sub> plus calcium versus calcium, a significant increase in BMD was not observed, suggesting vitamin D<sub>3</sub> is of little benefit in calcium-replete postmenopausal women. Vitamin D<sub>3</sub> alone versus placebo did not show significant increases in BMD, except in one trial that noted an increase in femoral neck BMD [93]. Only a few previous trials reported the impact of baseline serum 25(OH)D concentrations on BMD, and in all these trials, a lower baseline serum 25(OH)D was not associated with a greater BMD increase. However, two recent large RCTs show that greater BMD increases are seen in individuals with baseline serum 25(OH)D concentrations <30 nmol/L. In a substudy of the Vitamin D Assessment (ViDA) study, Reid et al. [94] studied whether monthly high-dose (100,000 IU) vitamin D supplementation given for 2 years influenced BMD in 452 New Zealand adults (mean age 69 years) or in those with low baseline serum 25(OH)D concentrations. The baseline serum 25(OH)D concentration in the total trial population was 58 nmol/L, and there were no significant changes in BMD at the lumbar spine or total body, but a reduction of the loss of femoral neck and total hip BMD by 0.5%. In the subgroup with the poorest vitamin D status at baseline (<30 nmol/L), BMD at the lumbar spine and femoral neck remained unchanged, whereas a 2% loss was recorded in the placebo group (Fig. 70.1).



**FIGURE 70.1** Effect of monthly high-dose vitamin D on bone density in community-dwelling older adults: Substudy of a randomized controlled trial (ViDA-BMD) [94]. Reproduced from Ref. [94], with permission from Wiley.

A second study recruited 305 postmenopausal women in late winter and randomized them to receive placebo, vitamin D 400 IU/day, or vitamin D 1000 IU/day over 1 year. Although bone loss from the hip was reduced by vitamin D 1000 IU/day only, there was no significant treatment effect of either dose at the spine [95]. However, in the trial participants with a baseline 25(OH)D concentration  $\leq 30$  nmol/L, significant treatment effects were seen at both the spine and hip, with no significant effects on BMD in those with baseline 25(OH)D concentrations above this level [95]. Similar findings were seen in the largest RCT, VITAL, in which there was no significant treatment effect of daily 2000 IU vitamin D on BMD or bone structure in the overall study. However, among participants with baseline-free vitamin D levels below the median ( $<14.2$  pmol/L), there was a slight increase in spine BMD (0.75% vs. 0%;  $P = .043$ ) and less bone loss from the total hip ( $-0.42\%$  vs.  $-0.98\%$ ;  $P = .044$ ) with vitamin D<sub>3</sub> treatment [96].

The daily dose of vitamin D should be at least 800 IU. There is evidence that daily doses  $\geq 4000$  IU are harmful to bone in females, but not in males. A 3-year randomized, double-blind trial investigated the effects of high-dose daily vitamin D supplementation on bone density and strength in 311 postmenopausal women and older men. Vitamin D supplementation with 4000 IU or 10,000 IU, compared with 400 IU daily, resulted in greater losses of total tibial volumetric BMD, measured by high-resolution peripheral quantitative computerized tomography (HR-pQCT) over 3 years in healthy vitamin D-sufficient females, but not males with similar trends being seen at the radius. There were no differential effects on bone strength [97].

A prior metaanalysis examined effects of calcium or calcium combined with vitamin D on BMD and fractures in men and women aged  $>50$  years [98]. Of the 23 trials ( $n = 41,419$ ) reporting BMD as an outcome, calcium and calcium in combination with vitamin D were associated with a reduced bone loss of 0.54% at the hip, and 1.19% at the spine. A positive treatment effect on BMD was evident in most studies; however, the treatment effect of vitamin D alone was not assessed.

Calcium and vitamin D supplementation during winter results in increases in spinal and femoral neck BMD, compared with decreases in those older men and women not receiving supplements [66]. One trial examined the effect of the combination of calcium and vitamin D on bone structure and bone turnover compared with calcium alone [99]. Three hundred and two elderly women with baseline serum 25(OH)D concentrations  $<24$  ng/mL (60 nmol/L) participated in a 1-year randomized, double-blind, placebo-controlled trial of 1000 mg calcium per day with either 1000 IU ergocalciferol (vitamin D<sub>2</sub>) or identical placebo. Baseline

calcium intake was 1100 mg/day, and serum 25(OH)D was 18 ng/mL (44 nmol/L); this increased only in the vitamin D group to 24 ng/mL (60 nmol/L) after 1 year. Total hip and total body BMD increased significantly, and procollagen type I intact N-terminal propeptide (P1NP) decreased by  $\sim 4\%$  during the study, with no difference between the treatment groups. In addition, there was no effect on active fractional intestinal calcium absorption, which fell equally in both groups by 15%–17%. Thus, ergocalciferol (vitamin D<sub>2</sub>) 1000 IU for 1 year had no beneficial effects on bone structure, bone formation markers, or intestinal calcium absorption over an additional 1000 mg of calcium.

In the same cohort, falls risk was measured every 6 months. Ergocalciferol (vitamin D<sub>2</sub>) 1000 IU combined with calcium reduced the risk of having at least one fall over 1 year by 39% compared with calcium supplementation alone [100]. When those who fell were grouped by either the season of first fall or the number of falls, ergocalciferol treatment reduced the risk of having the first fall in winter and spring (ergocalciferol group, 25.2%; control group, 35.8%; OR, 0.55), but not in summer and autumn, and reduced the risk of having one fall by 50%, but not the risk of multiple falls. Thus, patients with a history of falling and vitamin D insufficiency benefited from vitamin D supplementation in addition to calcium, which was associated with an overall 19% reduction in the relative risk of falling, mostly in winter. However, a post hoc analysis of a large RCT of 5108 individuals treated with 100,000 IU vitamin per month versus placebo (ViDA study) for 2.5–4.2 years showed no difference in falls in this healthy, ambulatory, adult population [101]. In a double-blind, placebo-controlled RCT (VITAL), 25,871 adults (mean age, 67.1 years) were randomly assigned to cholecalciferol (2000 IU/day) and/or omega-3 fatty acids (1 g/day) or respective placebos in a  $2 \times 2$  factorial design [102]. The numbers of participants with  $\geq 2$  falls were similar between active and placebo groups. Over 5 years, there were no differences in the proportion having  $\geq 2$  falls, or falls resulting in a doctor visit, or a hospital visit between groups. The results also did not differ between those with baseline serum 25(OH)D concentrations  $<$  or  $\geq 50$  nmol/L, although the proportion of the study population with serum vitamin D  $< 50$  nmol/L was low (14.8%). Other studies show either no effect or an increased risk of falls with vitamin D. A placebo-controlled RCT of community-dwelling Finnish women aged 70–80 years with a baseline 25(OH)D concentration of 32 nmol/L showed vitamin D (800 IU/day) with or without exercise showed no effect of vitamin D on the falls rate [103]. A smaller 1-year dose-ranging study in postmenopausal women selected for vitamin D deficiency indicated in a secondary analysis a U-shaped curve of effects of the seven combined vitamin D doses on fall



risk, compared with the placebo group [104]. In older community-dwelling adults aged  $\geq 70$  years, annual bolus doses of 500,000 IU and monthly doses of 60,000 IU have been shown in prior studies to increase falls in community-dwelling older adults (see the following).

A 5-year randomized, controlled, double-blind trial of 120 community-dwelling women aged 70–80 years compared 1200 mg/day calcium with placebo vitamin D (Ca group) or with 1000 IU/day ergocalciferol (vitamin D<sub>2</sub>) (CaD group), or double placebo (control) [105]. Hip BMD was preserved in both intervention groups, but not in controls at year 1 and maintained in the CaD group only over the long term at years 3 and 5. At year 1, compared with controls, the Ca and CaD groups had lower bone turnover markers. At 5 years, the suppression of bone formation and bone resorption markers was maintained only in the CaD group, and this decrease was also associated with reductions in PTH at 3 and 5 years compared with controls. Thus, although the combination of calcium with vitamin D had no additional effect over calcium alone in the short term (at 1 year), continuing skeletal benefits appear to be greater with this combination over the long term (up to 5 years).

Another study examined effects of calcium and vitamin D–fortified milk (1000 mg of calcium plus 800 IU of vitamin D<sub>3</sub>) compared with normal diet over 24 months in older men [106]. After 2 years, the mean percent decrease in BMD was 0.9%–1.6% less in the milk supplementation compared with control group at the femoral neck, total hip, and radius. There was a greater increase in lumbar spine BMD in the milk supplementation group after 12 and 18 months, but not after 2 years. Serum 25(OH)D increased and PTH decreased in the milk supplementation relative to control group after both years of the study. After a further 18 months of follow-up, during which neither group received calcium- and vitamin D<sub>3</sub>–fortified milk, the net beneficial effects of fortified milk on femoral neck and radius BMD at the end of the intervention were sustained [107]. However, there were no lasting benefits at the lumbar spine, suggesting the possibility of sustained skeletal benefits on cortical bone after withdrawal.

## 6.1 Conclusion

Treatment with the combination of calcium and vitamin D prevents bone loss and results in small increases in BMD at most sites. For long-term maintenance of BMD up to 5 years, the combination of calcium and vitamin D appears to be better than calcium alone. These skeletal benefits of calcium and vitamin D may be maintained at some, but not all, skeletal sites after withdrawal. However, the data also suggest that vitamin D

alone is effective in maintaining or increasing BMD over 1–2 years in individuals with a serum 25(OH)D concentration  $< 30$  nmol/L. There is evidence that daily vitamin D doses of  $\geq 4000$  IU are harmful for bone in postmenopausal women. The addition of vitamin D to calcium is also likely to reduce the risk of falling, particularly in winter, in patients with a history of falling and vitamin D insufficiency. This may result in a reduction of falls-related fractures. This has not been seen with vitamin D alone and higher bolus doses of vitamin D, which appear to increase falls in older individuals.

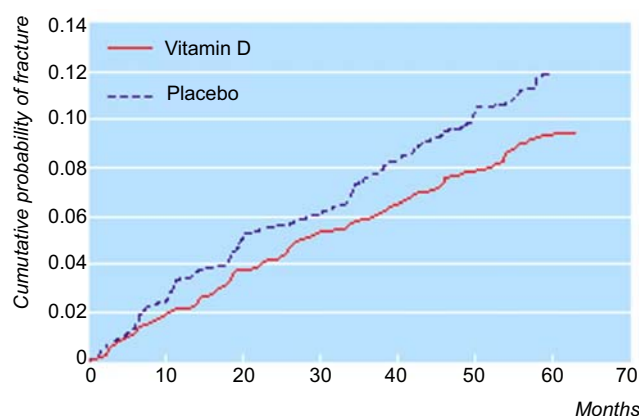
## 7. Primary fracture prevention with vitamin D or calcium and vitamin D

Primary fracture prevention is the most important potential role for vitamin D supplementation. However, until recently, most studies have been performed using a combination of calcium and vitamin D supplementation rather than vitamin D alone. The quality of many studies has also been reduced first by poor long-term compliance with study medications, and rates have mostly ranged from 55% to 75%, and second by a high degree of variability of dosing regimens.

### 7.1 Effect of vitamin D alone on fractures

The study showing the most benefit of vitamin D supplementation alone on fracture reductions was by Trivedi et al. [108]. They studied 2037 men and 649 women aged 65–85 years, predominantly doctors, living in the general community in a randomized, double-blind, controlled trial of 100,000 IU oral vitamin D<sub>3</sub> (cholecalciferol) or matching placebo every 4 months over 5 years. After 5 years, 268 men and women had incident fractures, of whom 147 had fractures in common osteoporotic sites (hip, wrist or forearm, or vertebrae). Relative risks in the vitamin D group compared with the placebo group were 0.78 for any fracture (Fig. 70.2) and 0.67 for first hip, wrist or forearm, or vertebral fracture. Four hundred and seventy-one participants died; however, the relative risk for total mortality in the vitamin D group compared with the placebo group was not significantly reduced (RR = 0.88).

Metaanalyses, however, show no overall effect of vitamin D alone on fracture risk. A Cochrane review of 53 RCTs in postmenopausal women or men over 65 years compared vitamin D supplements with placebo or no intervention or calcium supplements [109]. Medication regimens varied from vitamin D<sub>3</sub> 700–800 IU daily. Results from 11 trials (27,693 participants) showed no significant reduction in incidence of new hip fracture (RR, 1.12; 95% CI, 0.98–1.29) and nonvertebral fractures



**FIGURE 70.2** Cumulative probability of any first fracture with 100,000 IU cholecalciferol every 4 months versus placebo over 5 years;  $P = .04$  [108]. Reproduced from Ref. [108], with permission from BMJ publishing group.

(RR, 1.05; 95% CI, 0.96–1.14) for vitamin D alone compared with placebo or no intervention. Four trials with 3021 participants compared vitamin D with calcium. There was no evidence of a statistically significant difference between vitamin D alone and calcium in the prevention of hip fracture (RR, 0.90; 95% CI, 0.61–1.32) or nonvertebral fractures (RR, 1.10; 95% CI, 0.91–1.33). There was evidence that vitamin D alone was less effective than calcium for the prevention of vertebral fracture (RR, 2.21; 95% CI, 1.08–4.53). Thus, there is no evidence for the effectiveness of vitamin D alone for prevention of fractures.

A good-quality systematic review (9 RCTs,  $n = 53,260$ ) investigated the need for calcium supplementation (500–1200 mg daily) in postmenopausal women and men receiving vitamin D (cholecalciferol 700–800 IU daily [6 RCTs] or 400 IU daily [3 RCTs]) for prevention of fractures [110]. Mean therapy duration was 20–84 months. Pooled results showed vitamin D alone was not associated with a reduction in risk of hip fracture (RR, 1.10; 95% CI, 0.89–1.36;  $P = .38$ ) or a reduction in risk of nonvertebral fractures (RR, 0.98; 95% CI, 0.83–1.16;  $P = .79$ ) compared with placebo. An indirect comparison of trials investigating vitamin D with calcium compared with those investigating vitamin D alone showed an RR of 0.75 (95% CI, 0.58–0.96;  $P = .02$ ) for hip fracture.

Another metaanalysis addressed the effect of vitamin D supplementation on all fractures in postmenopausal women and men aged 50 years or older [92]. The pooled results for all fractures included 10 double-blind RCTs and 3 open study–design trials ( $n = 58,712$ ) and did not show a significant reduction of fractures with vitamin D alone (pooled odds ratio, 0.90; 95% CI, 0.81–1.02). In this report, the benefit of vitamin D depended on additional calcium and was also primarily seen in institutionalized individuals.

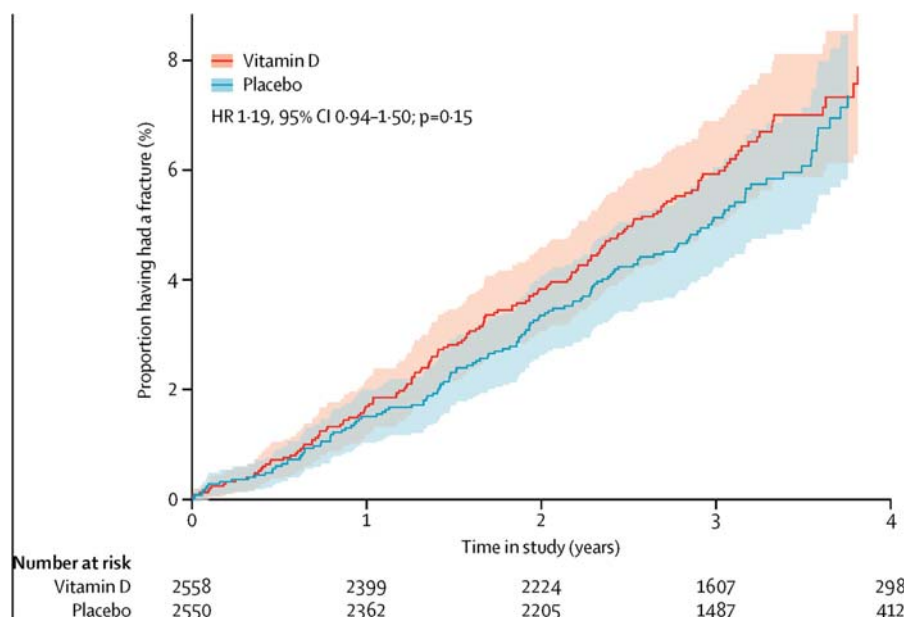
Such negative results might be explained by heterogeneity between studies in doses of vitamin D. In a further metaanalysis of 12 double-blind RCTs among individuals aged 65 years or older, the antifracture efficacy of supplemental vitamin D increased significantly with either a higher received dose or a higher achieved 25(OH)D level for any nonvertebral fractures and for hip fractures [84]. No fracture reduction was observed for a received daily dose of  $\leq 400$  IU, whereas a higher received daily dose of 482–770 IU of supplemental vitamin D reduced nonvertebral fractures by 20% and hip fractures by 18%. Subgroup analyses for the prevention of nonvertebral fractures with the higher received dose suggested possibly better fracture reduction with cholecalciferol compared with ergocalciferol, whereas additional calcium did not further improve antifracture efficacy. Nonvertebral fracture reduction with the higher received dose was significant among all subgroups by age and dwelling, including younger individuals aged 65–74 years and those living in the community.

Using individual patient data from seven major randomized trials of vitamin D with calcium or vitamin D alone, yielding a total of 68,517 participants with a mean age of 70 years (14.7% men), the results indicated that vitamin D given alone in daily doses of 10–20  $\mu\text{g}$  (400–800 IU) was not effective in preventing fractures [111]. However, trials using vitamin D with calcium showed a reduced overall risk of fracture (HR, 0.92; 95% CI, 0.86–0.99;  $P = .025$ ) and hip fracture (all studies: 0.84; 0.70–1.01,  $P = .07$ ; studies using 10  $\mu\text{g}$  (400 IU) of vitamin D given with calcium: 0.74; 0.60–0.91,  $P = .005$ ). No interaction was found between fracture history and treatment response, nor was there any interaction with age, sex, or HRT.

Three recent large trials (DO-HEALTH, ViDA, VITAL) have published data on incident fracture in older community-dwelling healthy adults treated with vitamin D or placebo. Data are also being analyzed in a third trial, which compared 60,000 IU vitamin D per month with placebo over 5 years (D-Health).

The 3-year DO-HEALTH study examined whether 2000 IU daily vitamin D, omega-3 fatty acids, and a strength-training exercise program, alone or in combination, reduced nonvertebral fractures among 2157 adults aged  $\geq 70$  years (mean age 74.9 years) without previous major health events. The mean baseline serum 25(OH)D concentration was 56 nmol/L (22.4 ng/mL) and increased to 94 nmol/L (37.6 ng/mL). There was no effect of any of the interventions on nonvertebral fractures [112].

The ViDA study compared monthly vitamin D 100,000 IU per month versus placebo for 2.5–4.2 years in 5108 healthy, ambulatory, adults and showed no difference in incident fractures (HR, 1.19; 95% CI, 0.94–1.50,  $P = .15$ ) [101] (Fig. 70.3). In a double-blind,



**FIGURE 70.3** Cox proportional hazards model of nonvertebral fractures recorded during follow-up with 100,000 IU cholecalciferol per month and placebo. Lines depict the proportion of participants having a fracture during follow-up, and shading represents the 95% CI. HR, hazard ratio [101]. Reproduced from Ref. [101], with permission from Lancet publishing group.

placebo-controlled RCT (VITAL), 25,871 adults were randomly assigned to cholecalciferol (2000 IU/day), and/or omega-3 fatty acids (1 g/day) or respective placebos in a  $2 \times 2$  factorial design [113] for 5 years. The mean baseline 25(OH)D level (16,757 participants) was  $30.7 \pm 10.0$  ng/mL (77 nmol/L). At baseline, 2.4% had 25(OH)D levels <12 ng/mL (30 nmol/L) and 12.9% had levels <20 ng/mL (50 nmol/L). The numbers of participants with incident total, nonvertebral, and hip fractures were similar between active and placebo groups (Fig. 70.4). There was also no effect of vitamin D on BMD or bone structure; however, in participants with baseline free 25(OH)D levels below the median, supplemental vitamin D<sub>3</sub> resulted in slight increases in spine and total hip BMD. From these two studies, vitamin D<sub>3</sub> supplementation did not result in a significantly lower risk of fractures than placebo among generally healthy midlife and older adults not selected for vitamin D deficiency, low bone mass, or osteoporosis. However, there was limited study power to examine effects of vitamin D on fractures in patients with vitamin D deficiency because of the low numbers of fractures in participants with vitamin D deficiency at baseline.

### 7.1.1 Conclusion

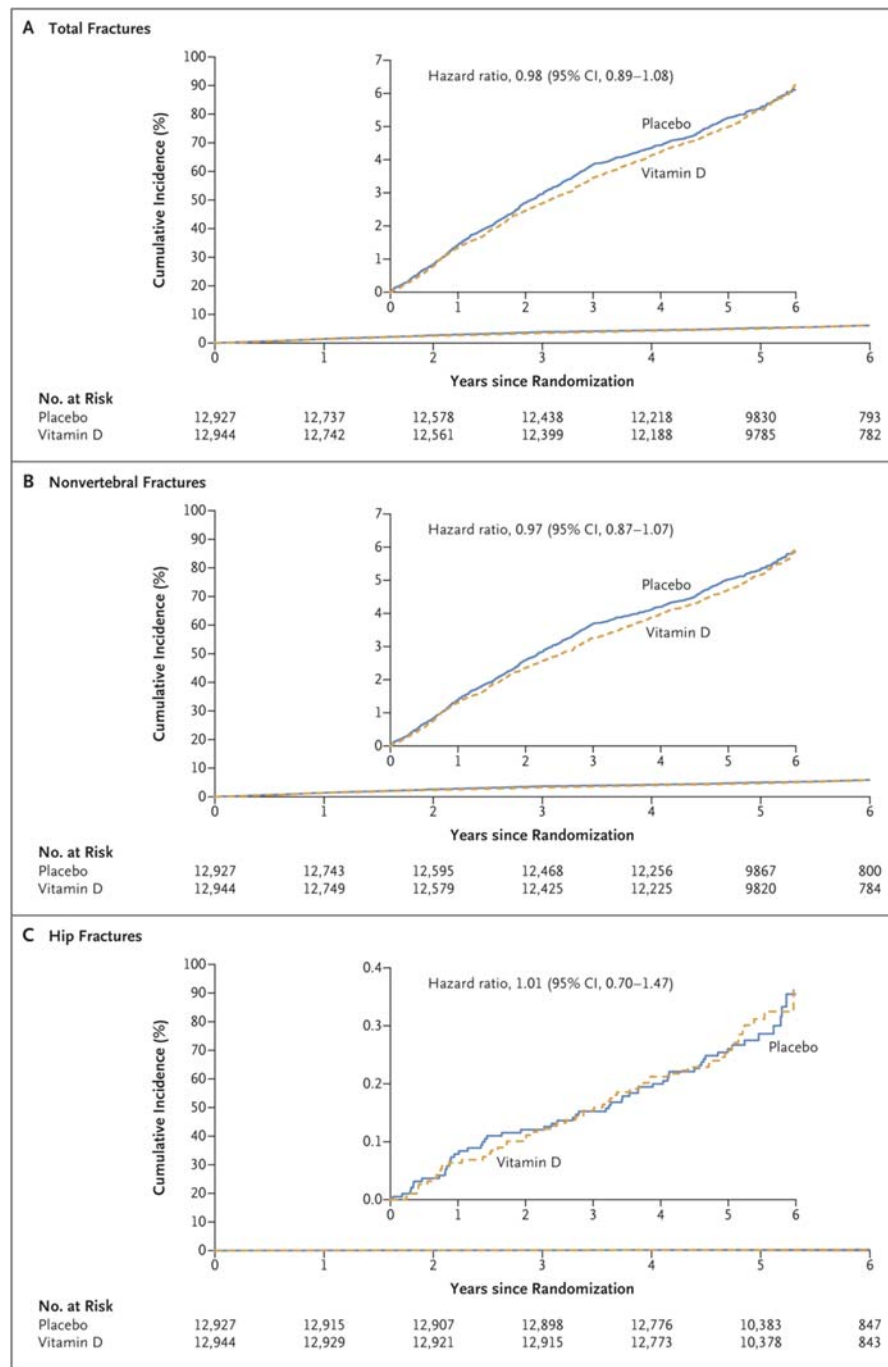
Overall evidence from several metaanalyses shows no effect of vitamin D alone on fracture risk. Although the effects of vitamin D on fracture risk may be masked both by heterogeneity of the received daily doses between studies, and the low numbers of included participants with vitamin D deficiency at baseline, the

evidence for vitamin D reducing the risk of nonvertebral and hip fractures is most compelling with the use of additional calcium in institutionalized individuals.

## 7.2 Single high annual or monthly doses of vitamin D

The first study investigating single large annual doses of vitamin D utilized an annual intramuscular injection of 150,000–300,000 IU vitamin D<sub>2</sub> (ergocalciferol) in 479 Finnish men and women aged >85 years, who were living in their own home and 320 subjects aged 75–84 years living in a home for aged people [114]. Although treatment allocation was not adequately randomized or blinded, the group of subjects administered intramuscular vitamin D had a significantly lower rate of all fractures. This was particularly marked for fractures of the upper limb but was not apparent for lower limb sites.

The second study was a randomized, double-blind, placebo-controlled trial of 300,000 IU intramuscular vitamin D<sub>2</sub> (ergocalciferol) injection or matching placebo every autumn over 3 years. Nine thousand four hundred and forty people (4354 men and 5086 women) aged  $\geq 75$  years were recruited from general practices in England [115]. Five hundred and eighty-five subjects had incident nonspine fractures (hip 110, wrist 116, ankle 37). HRs for fracture in the vitamin D group were 1.09 (95% CI, 0.93–1.28) for any first fracture, 1.49 (95% CI, 1.02–2.18) for hip, and 1.22 (95% CI, 0.85–1.76) for wrist. There was no effect on falls:



**FIGURE 70.4** Cumulative incident total, nonvertebral, and hip fractures with 2000 IU vitamin D daily and placebo [113]. Reproduced from Ref. [113], with permission from New England Journal of Medicine.

HR, 0.98 (0.93–1.04). When the analyses were confined to fractures at the wrist, hip, or both sites, fracture incidence tended to be higher in the vitamin D–treated arm when compared with placebo. However, these differences were not statistically significant. There was, however, a significant interaction between sex and treatment group on the risk of any nonvertebral fracture, such that a detrimental effect of vitamin D was seen among

women, but not among men ( $P < .03$ ). This implies annual intramuscular vitamin D<sub>2</sub> injections may increase the risk of nonvertebral fractures in women.

These data were confirmed in a double-blind, RCT of annual doses of oral cholecalciferol 500,000 IU, given in autumn or winter, versus placebo performed by Sanders et al. [116]. Two thousand two hundred and fifty-six community-dwelling women, aged  $\geq 70$  years, were



randomly assigned to receive oral cholecalciferol or placebo each autumn to winter for 3–5 years. Fractures were radiologically confirmed. Women in the cholecalciferol (vitamin D) group had 171 fractures versus 135 in the placebo group; the fall rate was 83.4 per 100 person-years in the vitamin D group, and 72.7 per 100 person-years in the placebo group (incidence rate ratio [IRR], 1.15,  $P = .03$ ). The IRR for fracture in the vitamin D group was 1.26 (95% CI; 1.00–1.59;  $P = .047$ ) versus the placebo group (rates per 100 person-years, 4.9 vitamin D vs. 3.9 placebo) (Fig. 70.5). A temporal pattern was observed in a post hoc analysis of falls. The IRR for falling in the vitamin D group versus the placebo group was 1.31 in the first 3 months after dosing and 1.13 during the following 9 months (test for homogeneity;  $P = .02$ ). Although not significant, the IRR for fractures was also increased in the vitamin D group versus the placebo group in the first 3 months compared with the following 9 months after dosing (RR, 1.53 vs. 1.18). The median baseline serum 25(OH)D was 49 nmol/L in this community-based study. In the vitamin D group, serum 25(OH)D concentrations increased at 1 month after dosing to  $\sim 120$  nmol/L and were  $\sim 90$  nmol/L at 3 months. The cause of the increased rate of falls or fractures after dosing is unknown but might relate to rapid fluxes in vitamin D metabolites or vitamin D–regulated genes or, perhaps, even to increased levels of physical activity.

A recent double-blind, randomized study has also raised concern about the safety of high monthly doses of vitamin D in older individuals [117]. Two hundred men and women with a prior fall were randomized to three monthly doses of vitamin D (24,000 IU vitamin D<sub>3</sub>, 60,000 vitamin D<sub>3</sub> and 24,000 vitamin D<sub>3</sub>, and

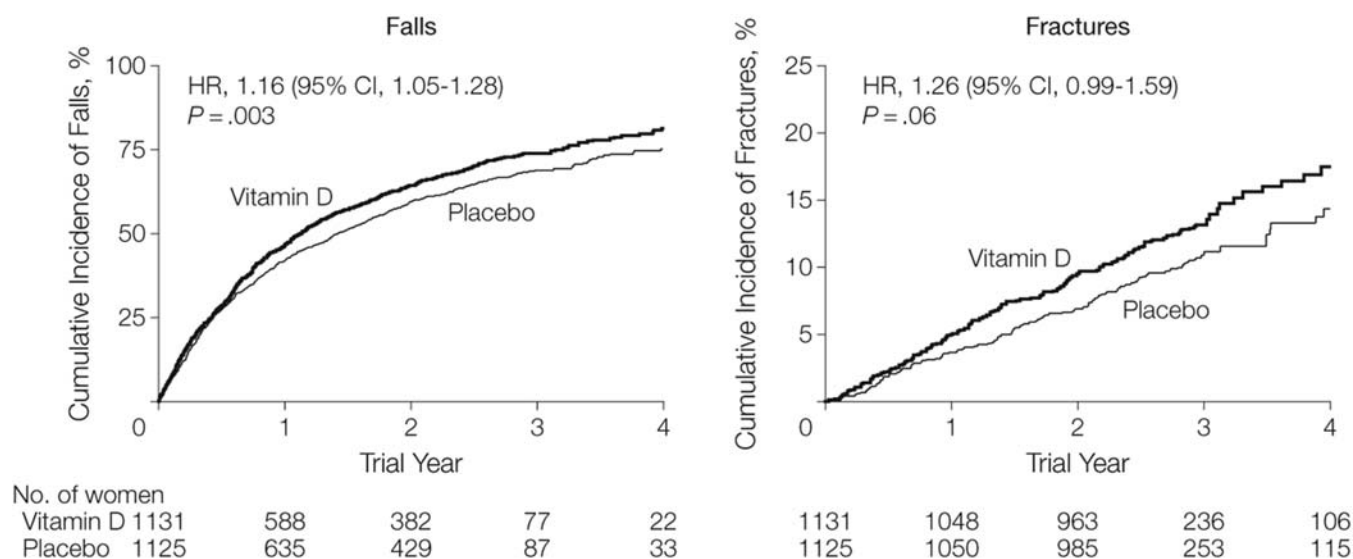
300  $\mu$ g calcidiol). Over 12 months of follow-up, there was a higher incidence of falls in the 60,000 IU vitamin D<sub>3</sub> and 24,000 vitamin D<sub>3</sub> and 300  $\mu$ g calcidiol groups, of 66.9% and 66.1%, respectively, versus 47.9% in the 24,000 IU group ( $P = .048$ ).

### 7.2.1 Conclusion

High annual or monthly doses of vitamin D are not recommended either to treat vitamin D deficiency or to prevent fractures in older women and men. In addition, the safety of high-dose vitamin D supplementation warrants further study, as the levels of 25(OH)D seen in these studies ( $\geq 48$  ng/mL or 120 nmol/L) may have had detrimental effects on falls and fractures in older men and women. In this regard, studies using either monthly doses of 50,000 IU cholecalciferol [118] or a loading dose of 10 daily doses of 50,000 IU cholecalciferol [119] achieved more modest increases in serum 25(OH)D to just above the optimal target range (75 nmol/L) at 3 months.

## 7.3 Primary fracture prevention with calcium and vitamin D

The pivotal study from which many metaanalyses on the effects of calcium and vitamin D on fractures derive their strength was performed in 3270 ambulatory elderly women (mean age 84 years) residing in institutional care (nursing homes or apartments) [120]. Supplementation with 1.2 g calcium and 800 IU cholecalciferol reduced hip fractures and nonvertebral fractures by 43% and 32%, respectively, and resulted in increases in hip BMD over 18 months (Fig. 70.5). The



**FIGURE 70.5** Increase in falls and fractures after an annual dose of 500,000 IU cholecalciferol [116]. CI, confidence interval; HR, hazard ratio. Reprinted from Ref. [116], with permission from the American Medical Association.

mean baseline serum 25(OH)D concentration was 16 ng/mL (40 nmol/L), meaning most women were vitamin D insufficient or deficient. The number needed to treat (NNT) to prevent a hip or nonvertebral fracture was 41 and 29, respectively. These data were confirmed in a later study performed by the same authors [121]. One study also showed calcium and vitamin D reduce fractures in community-dwelling healthy men and women. In 176 men and 213 women with a mean age of 70–72 years and having mean calcium intakes of 673–798 mg per day, 500 mg calcium and 700 IU cholecalciferol reduced nonvertebral fractures by 50% over 3 years and resulted in increases in hip, spine, and total body BMD [122]. The NNT to prevent a nonvertebral fracture was 15.

The largest study of calcium and vitamin D used daily doses of cholecalciferol (400 IU) that would now be considered suboptimal, and the mean baseline calcium intake in this study was also high (1150 mg/day) [92]. Thirty-six thousand two hundred and eighty-two postmenopausal women, aged 50–79 years, were randomly assigned to receive 1000 mg calcium with 400 IU cholecalciferol per day, or placebo. Participants receiving calcium plus vitamin D supplementation had a nonsignificant decrease in hip fracture risk (HR, 0.88; 95% CI, 0.72–1.08), HR of 0.90 for clinical spine fracture (0.74–1.10), and HR of 0.96 for total fractures (0.91–1.02). The risk of renal calculi increased with calcium plus vitamin D (HR, 1.17; 1.02–1.34). Censoring data from women when they ceased to adhere to the study medication indicated a reduction in hip fracture risk by 29% (HR, 0.71; 0.52–0.97).

Another study of 3432 community-dwelling northern Finnish women aged 65–71 years assessed incident fractures in women randomly assigned to receive 1000 mg calcium with 800 IU of cholecalciferol per day or placebo over 3 years [123]. The risk of any fracture decreased in the vitamin D and calcium group by 17% (95% CI, 0.61–1.12), and the risk of any nonvertebral fracture decreased by 13% (0.63–1.19). The risk of distal forearm fractures decreased by 30% (0.41–1.20), and the risk of any upper extremity fractures decreased by 25% (0.49–1.16), whereas the risk of lower-extremity fractures did not change. However, none of these effects reached statistical significance. This study provides further evidence that vitamin D and calcium supplementation does not prevent fractures in older community-dwelling postmenopausal women.

## 8. Metaanalyses

Metaanalyses indicate mixed evidence for the impact of oral calcium and vitamin D supplementation on reduction of fractures outside institutionalized settings.

One good-quality systematic review (29 studies, 63,867 individuals, 92% female) reported on the effect of calcium supplementation (alone or in combination with vitamin D) in doses of 1000–1200 mg in adults aged over 50 years [98]. Overall, calcium supplementation was associated with a 12% reduction in risk of any fracture (RR, 0.88; 95% CI, 0.83–0.95), with similar reduction in risk of fractures in trials using calcium supplements alone (10% risk reduction) and those where calcium was administered in combination with vitamin D (13% risk reduction). The estimated NNT to prevent one fracture over 3.5 years was 63 in the overall population. For individuals who were elderly, lived in institutions, had a low body weight, had a low calcium intake (less than 700 mg per day), or were at a higher baseline risk of fracture, the NNT to prevent one fracture over 3.5 years was 30. The fracture risk reduction was significantly greater (24%) in trials in which the compliance rate was high ( $P < 0.0001$ ). The treatment effect was better with daily calcium doses  $\geq 1200$  mg than with doses  $< 1200$  mg (0.80 vs. 0.94;  $P = 0.006$ ), and with daily vitamin D doses of  $\geq 800$  IU than with doses  $< 800$  IU (0.84 vs. 0.87;  $P = 0.03$ ). People with low serum 25(OH)D concentrations ( $< 25$  nmol/L) had a greater risk reduction compared with those whose serum 25(OH)D was normal (RR, 0.86 vs. 0.94); however, this result was not significant ( $P = .06$ ).

A good-quality systematic review (9 RCTs,  $n = 53,260$ ) investigated the need for calcium supplementation 500–1200 mg per day in postmenopausal women and men receiving vitamin D [either cholecalciferol 700–800 IU per day (6 RCTs) or 400 IU daily (3 RCTs)] for prevention of fractures [107]. Mean therapy duration was 20–84 months. Results (6 RCT,  $n = 45,509$ ) of vitamin D in conjunction with calcium supplements compared with placebo or no treatment showed a significant reduction in risk of both hip fracture (RR, 0.82; 95% CI, 0.71–0.94) and nonvertebral fracture (0.88; 0.78–0.99). The NNT to prevent one hip fracture over 24–84 months was 276, and NNT to prevent one nonvertebral fracture was 72. An indirect comparison of trials investigating vitamin D with calcium compared with those investigating vitamin D alone showed a significant risk reduction in hip fracture (RR, 0.75; 0.58–0.96).

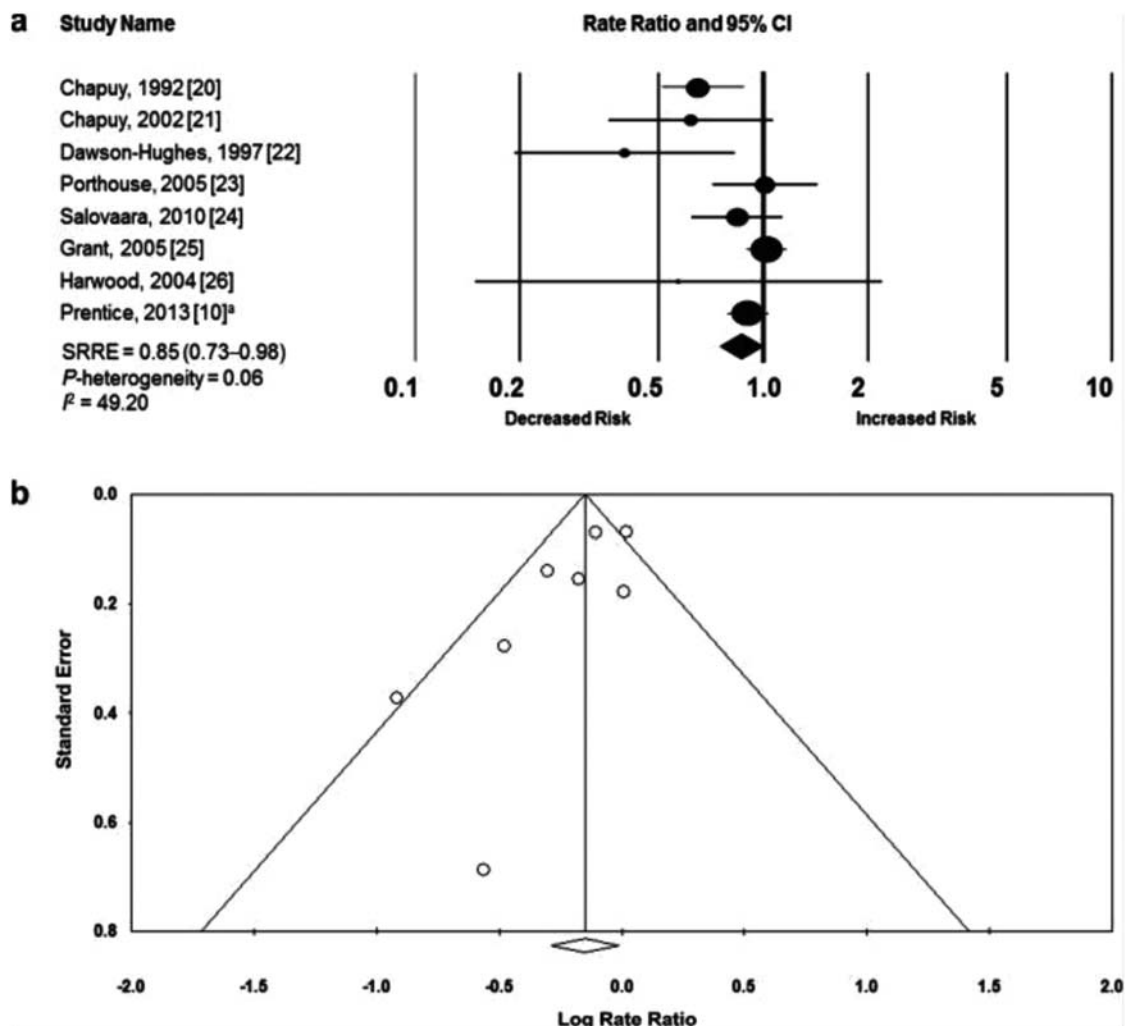
A Cochrane review of 53 RCTs in postmenopausal women or men over 65 years compared calcium and vitamin D supplements to placebo, or no intervention [109]. Medication regimens varied from calcium 1000 to 1200 mg and vitamin D<sub>3</sub> 700 to 800 IU daily. Results from nine trials (49,853 participants) showed high quality evidence that vitamin D plus calcium results in a small reduction in hip fracture risk (RR, 0.84; 95% CI, 0.74–0.96;  $P = 0.01$ ). In low-risk populations (residents in the community), this equates to one fewer hip fracture

per 1000 older adults per year. In high-risk populations (institutionalized elderly), this equates to nine fewer hip fractures per 1000 older adults per year. There is also high-quality evidence from eight trials with 10,380 participants that vitamin D plus calcium is associated with a statistically significant reduction in incidence of new nonvertebral fractures (RR, 0.86; 95% CI, 0.78–0.96). However, there is only moderate quality evidence of an absence of a statistically significant preventive effect on clinical vertebral fractures. There is high-quality evidence that vitamin D plus calcium reduces the risk of any type of fracture (10 trials, 49,976 participants; RR, 0.95; 95% CI, 0.90–0.99).

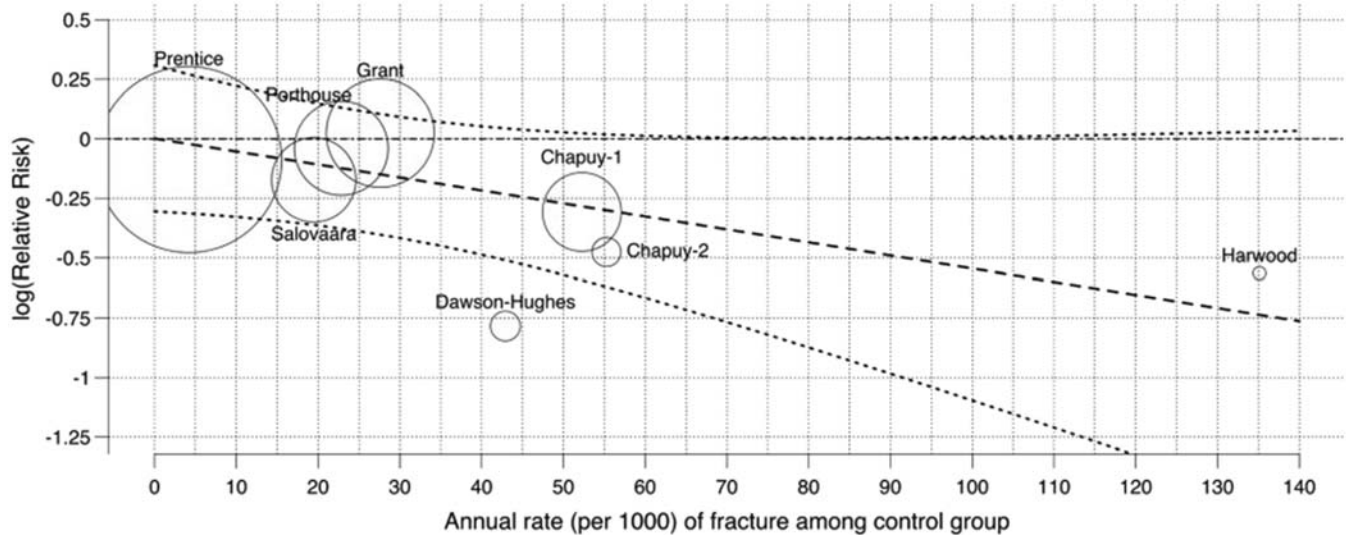
A metaanalysis from the National Osteoporosis Foundation included eight studies with 30,970 participants who met criteria for inclusion in the primary analysis, with 195 hip fractures and 2231 total fractures [124]. It showed vitamin D and calcium supplementation

produced a statistically significant 15% reduced risk of total fractures (RR, 0.85; 95% CI, 0.73–0.98) (Fig. 70.6) and a 30% reduced risk of hip fractures (RR, 0.70; 95% CI, 0.56–0.87). A limitation was that this study utilized data from subgroup analysis of the Women's Health Initiative.

An alternative analysis of these data using a Bayesian approach found that the decrease in fracture risk was less certain, with an overall 13% reduction (RR, 0.87; 95% credible interval, 0.68–1.02) [125]. However, the heterogeneity between studies could be partially explained by differences in background risk between studies (Fig. 70.7). For example, in patients with a moderate background annual risk for fracture, say 50 per 1000 older adults, only 84 individuals need to be treated with vitamin D and calcium to prevent one fracture, whereas that number would be much higher for patients with a low background risk. This implies difficulty in



**FIGURE 70.6** Calcium plus vitamin D supplementation versus placebo and total fracture. (A) Metaanalysis of 8 RCTs; (B) funnel plot of standard error by log rate ratio for calcium plus vitamin D supplementation and risk of total fracture [124]. CI, confidence interval; SSRE, summary relative risk estimate. Reproduced from Ref. [124], with permission from Springer.



**FIGURE 70.7** Relationship between effect size (log relative risk) and background risk of fracture [125]. Reproduced from Ref. [125], with permission from Springer.

making recommendations for reducing fractures with vitamin D and calcium at a population level.

A further metaanalysis of 33 randomized trials comparing supplements with placebo or no treatment involving 51,145 community-dwelling adults aged >50 years found no significant associations between calcium, vitamin D, or combined calcium and vitamin D supplements and the incidence of hip, nonvertebral, vertebral, or total fractures [126]. However, the Chapuy studies [120,121] of calcium and vitamin D were excluded, while the large RECORD trial of secondary fracture prevention (see the following) was included, limiting the inferences drawn from this metaanalysis.

A systematic umbrella review of metaanalyses of controlled trials examined 13 systematic reviews/metaanalyses (SR/MAs) on vitamin D and calcium (Ca/D) and 19 SR/MAs on vitamin D alone, compared with placebo/control [127]. Only 2 out of 10 SRs/MAs on Ca/D were of moderate quality. The combination of calcium and vitamin D reduced the risk of hip fractures in 8 of 12 SRs/MAs relative risk ([RR] 0.61–0.84), and any fractures in 7 of 11 SR/MAs (RR 0.74–0.95). Importantly, no fracture risk reduction was noted in SRs/MAs exclusively evaluating community-dwelling individuals or in those on vitamin D alone compared with placebo/control. The authors concluded individual participant data metaanalyses of patients on Ca/D with sufficient follow-up periods, and subgroup analyses, would unravel determinants for a beneficial response to supplementation.

There is evidence for an effect of vitamin D supplementation for the primary prevention of minimal trauma fractures, including hip fractures, in postmenopausal women and older men, but only when combined

with calcium supplements. The effect size for primary fracture prevention is likely to be larger for those with a higher background fracture risk, including those who have the lowest serum levels of 25(OH)D (<25 nmol/L) and in institutionalized patients. In women and men aged >50 years, the combination of vitamin D with calcium, but not vitamin D alone, has modest effects in preventing any nonvertebral and hip fractures ranging from 5% for any fractures in the Cochrane review [109] to 30% for hip fractures in the National Osteoporosis Foundation metaanalysis [124]. The daily dose of vitamin D should be at least 800 IU (20 µg). High single annual or monthly doses of vitamin D may increase falls and, possibly, fractures in older men and women and are, therefore, not recommended.

## 9. Safety

### 9.1 Vitamin D

Vitamin D alone is safe at daily doses of 2000 IU per day and is likely to be safe up to 4000 IU per day. However, the recent studies [116,117] showing an increased risk for falls following an annual dose of 500,000 IU, a monthly dose of either 60,000 IU or 24,000 IU vitamin D<sub>3</sub> when combined with 300 µg calcidiol (25(OH)D), means the safety of high-dose vitamin D supplementation warrants further study (see before).

### 9.2 Calcium and vitamin D

The safety of the combination of calcium and vitamin D has not been comprehensively assessed; however,



concern has recently been raised regarding the safety of calcium supplements. One RCT ( $n = 1471$ ) [128] and one metaanalysis using either individual patient or trial data [129] have reported on cardiovascular (CV) adverse events associated with calcium supplements compared with placebo or calcium and vitamin D in elderly, postmenopausal women. In the trial, there was no significant difference between groups in the risk of any CV event (angina, chest pain, myocardial infarction [MI], or sudden death), risk of stroke, or risk of sudden death. The risk of MI was not significant between groups for a number of validated events (RR, 1.49; 95% CI, 0.86–2.57). For the primary endpoint (risk of MI, stroke, or sudden death), there was no significant difference in the number of validated events (RR, 1.21; 0.84–1.74); however, the rate ratio showed a significant increase associated with calcium (RR, 1.43; 1.01–2.04;  $P = .043$ ).

In the metaanalysis of individual patient data, the risk of MI was increased (HR, 1.31; 95% CI, 1.02–1.67;  $P = .035$ ) [129]. The metaanalysis of trial level data showed similar results, with an increased incidence of MI in those allocated to calcium (RR, 1.27; 1.01–1.59;  $P = .038$ ) [128]. However, it should be noted that all trials were designed to assess the effect of calcium on BMD and the power to detect a clinical effect for CV outcome measures is not reported. In addition, it remains unclear whether the addition of vitamin D might attenuate the proposed adverse CV effects of calcium supplements. Calcium supplement use has also been associated with an increased risk of incident coronary artery calcification (RR, 1.20; 95% CI, 1.7–1.39); however, in the same study, high total calcium intakes (diet and supplements) were associated with a decreased risk of incident atherosclerosis [130].

### 9.2.1 Secondary fracture prevention with vitamin D or calcium and vitamin D

Either vitamin D alone or the combination of calcium and vitamin D is ineffective in reducing fractures in patients who have already had a minimal trauma fracture. A large study of 5292 women and men (15%) aged  $>70$  years with prevalent minimal trauma fractures examined secondary fracture prevention using 800 IU cholecalciferol (vitamin D<sub>3</sub>) per day versus 1000 mg calcium per day, or the combination of calcium and vitamin D<sub>3</sub>, or placebo over 2–5.2 years [131]. Thirteen percent of participants had a new minimal trauma fracture, 183 (26%) of which were hip fracture. The incidence of new minimal trauma fractures did not differ significantly between participants allocated calcium and those who were not (HR, 0.94; 95% CI, 0.81–1.09); between participants allocated vitamin D<sub>3</sub> and those who were not (1.02; 0.88–1.19); or between those allocated combination treatment and those assigned placebo (HR for interaction term, 1.01;

0.75–1.36). The groups did not differ in the incidence of all-new fractures, fractures confirmed by radiography, hip fractures, death, number of falls, or quality of life. This trial was notable for low adherence (54.5%) with tablets at 2 years and the ability of patients to start other antiosteoporotic therapy during the study. Compliance with tablets containing calcium was also significantly lower (difference: 9.4%; 6.6–12.2), partly because of gastrointestinal symptoms.

In a primary care, nurse-led study of 3314 women aged  $\geq 70$  years with one or more risk factors for hip fracture, including a previous fracture, daily oral supplementation with 1000 mg calcium and 800 IU cholecalciferol was given for 2 years in an open randomized trial [132]. Adherence with therapy at 2 years was low (55%). After a median follow-up of 25 months (range 18–42 months), clinical fracture rates were lower than expected in both groups but did not significantly differ for all clinical fractures (HR, 1.01; 95% CI, 0.71–1.43). The odds ratio for hip fracture was 0.75 (0.31–1.78). The odds of a woman having a fall at 6 and 12 months also did not differ between groups.

## 9.3 Conclusion

Despite the limitation of poor adherence in both studies, there is no evidence that calcium or vitamin D, either alone or in combination, is effective in reducing fractures in older women and men with preexisting minimal trauma fractures. In these individuals, antiosteoporosis drugs should be used instead. Interestingly, in women treated with commonly used antiresorptive drugs, treatment responses are improved in those with optimal serum 25(OH)D levels. In an Italian study, 1515 women with postmenopausal osteoporosis treated with antiresorptive drugs (alendronate, risedronate, raloxifene) for 13.1 months with an adherence rate  $>75\%$  were classified as vitamin D deficient ( $<20$  ng/mL or 50 nmol/L) ( $n = 453$ ) or replete [133]. Vitamin D-deficient or Vitamin D-replete subjects differed significantly in annualized spine and hip BMD changes adjusted for confounding factors. One hundred and fifty-one patients suffered from a new incident clinical fracture. The adjusted odds ratio for incident fractures in vitamin D-deficient compared with vitamin D-replete women was 1.77 (1.20–2.59;  $P = 0.004$ ).

Thus, a target serum level  $\geq 20$  ng/mL or 50 nmol/L should be aimed for in women and men on antiresorptive drugs to optimize skeletal responses. Most patients will require 800–2000 IU cholecalciferol per day to achieve these levels [134]. In addition, such levels will minimize the risk of hypocalcemia, particularly following parenteral antiresorptive therapy [135,136], and may reduce the severity of the acute-phase reaction

commonly seen after the first intravenous infusion of zoledronic acid [137].

## 10. Effects of active vitamin D analogs on fractures

There is no evidence that related vitamin D compounds (analogs) have advantages in terms of effectiveness or reduced incidence of adverse effects compared with vitamin D. Four small trials of alfacalcidol, of which three were in Japan by the same author in patients with neurological diseases [138–141], suggest that alfacalcidol was effective in reducing the incidence of hip fractures in older people with and without preexisting osteoporotic fractures (4 trials, 371 participants, RR, 0.18; 95% CI, 0.05–0.67). Positive results from these small studies need to be confirmed by other investigators in larger studies. Other small studies that examined effects on fractures at other skeletal sites, which compared alfacalcidol with calcium (with or without vitamin D), or alfacalcidol and calcium with calcium, were inconclusive [142].

Two trials compared calcitriol with calcium [143,144]. Overall, there was no statistically significant effect on the incidence of nonvertebral fractures (2 trials,  $n = 663$  participants; RR, 1.19; 95% CI, 0.09–15.77) or vertebral deformities (2 trials,  $n = 556$  participants; RR, 1.69; 95% CI, 0.25–11.28). In Tilyard et al. [144], the duration of treatment was critical. At the end of 1 year, no effect could be shown. Fewer vertebral deformities occurred in the calcitriol group during the second year (RR, 0.47; 95% CI, 0.26–0.87), and during the third year (RR, 0.28; 95% CI, 0.15–0.52). Thus, the effect of calcitriol in fracture prevention is unclear, with the best evidence for effectiveness coming from the trial where vertebral deformities were significantly reduced only in the second and third years. However, the use of calcitriol was associated with a statistically significant increase in the risk of hypercalcemia, so it is not recommended as a first-line drug to treat osteoporosis.

### 10.1 The anabolic vitamin D analogs, 2MD

Most antiosteoporosis drugs, including active vitamin D analogs, act by inhibiting bone resorption. 2MD (2-methylene-19-nor-(20S)-1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>) is a novel vitamin D analog that is a potent bone formation stimulating drug in vitro and also in vivo in an ovariectomized rat model [145]. However, in a randomized, double-blind, placebo-controlled study of osteopenic women, although daily oral treatment with 2MD caused a marked increase in markers of bone formation, it did not significantly increase BMD [146]. In this study, 2MD

also caused a marked increase in bone resorption, so the predominant effect of 2MD was to stimulate bone remodeling. This critical difference between the preclinical and clinical studies could reflect underlying differences in bone metabolism, including continuing skeletal modeling in the rat.

### 10.2 The vitamin D analogs, eldecalcitol

Eldecalcitol is an analog of 1,25(OH)<sub>2</sub>D<sub>3</sub> that in a double-blind, placebo-controlled trial with vitamin D supplementation in patients with osteoporosis increased lumbar spine BMD over 12 months in a dose-dependent manner. There was also a lower incidence of hypercalcemia in the 0.75  $\mu$ g/day group than in the highest dose group (1  $\mu$ g/day) [147]. In a 3-year double-blind, active comparator superiority trial, 1054 patients with osteoporosis were randomized to receive daily oral 0.75  $\mu$ g eldecalcitol or 1.0  $\mu$ g alfacalcidol to prevent fractures due to osteoporosis. The primary endpoint was reduction in vertebral fractures. After 3 years of treatment, the risk of vertebral fractures was 26% lower with eldecalcitol (absolute risk reduction (ARR) = 4.1%). Eldecalcitol also reduced bone remodeling rate and increased BMD more than alfacalcidol. The secondary endpoint of a reduced incidence of nonvertebral fractures was achieved predominantly due to a reduction in fractures of the distal radius by 71% compared with alfacalcidol (ARR 2.5%). However, increases in serum and urinary calcium were higher in the eldecalcitol group [148] and occurred in 21.0% and 25.6% of participants, respectively.

## 11. Large randomized controlled trials of vitamin D supplementation

At least seven large ( $n \geq 1000$ ) RCTs of vitamin D supplementation with a variety of nonskeletal primary outcomes have recently been performed or are ongoing. None target population groups likely to have low vitamin D levels and have recruited low numbers of participants with baseline 25(OH)D levels <25 nmol/L, in whom vitamin D is likely to be most effective [149] (Table 70.1). In addition, some studies are using doses equivalent to 60,000 IU vitamin D<sub>3</sub> per month, so it will be important to also examine safety outcomes carefully, including falls.

The D-Health Trial is an Australia randomized placebo-controlled trial, with planned intervention for 5 years and a further 5 years of follow-up through linkage with health and death registers. Two thousand one hundred and thirty-five participants aged 65–84 years are being treated with monthly oral doses of 60,000 IU

**TABLE 70.1** Proportions of participants below serum 25(OH)D concentrations associated with vitamin D insufficiency and deficiency at baseline in recent large studies.

Study	Baseline serum 25(OH)D	Serum 25(OH)D <25 nmol/L	Serum 25(OH)D <50 nmol/L
	Mean (SD)	N (%)	N (%)
ViDA	63 (24)	161 (3%)	1,534 (30%)
VITAL	77 (25)	621 (2.4%) <sup>a</sup>	3,337 (12.9%) <sup>a</sup>
D-health	80 (25)	298 (1.4%) <sup>a</sup>	2,558 (12%) <sup>a</sup>
Emerging RF collaboration		15,637 (4%)	

<sup>a</sup>Measured in a subset or calculated.

of cholecalciferol or placebo. The primary outcome is all-cause mortality. Secondary outcomes are total cancer incidence and colorectal cancer incidence. Other outcomes include falls and fractures [150]. Vitamin D had no effect on falls (odds ratio [OR], 1.02; 95% CI, 0.95 to 1.10). There was, however, a significant interaction with body mass index (BMI) (P-interaction = 0.001), with vitamin D increasing falls risk among people with BMI <25 kg/m<sup>2</sup> (OR, 1.25; 95% CI, 1.09–1.43) [151].

## 12. Mendelian randomization studies

Mendelian randomization studies use genetic markers associated with variations in 25(OH)D identified by genome-wide association studies (GWAS). A limitation of randomized trials is that they typically investigate short-term to medium-term interventions in a risk factor. However, for most diseases, it is probable that the benefit of short-term interventions in a risk factor will be less substantial than lifelong changes, which will be identified in Mendelian randomization studies. The results from Mendelian randomization studies estimating dose–response relationships for vitamin D with coronary heart disease, stroke, and all-cause mortality confirm earlier cohort studies showing nonlinear associations with measured 25(OH)D [152]. A recent genome-wide association study identified 143 independent loci associated with serum 25(OH)D concentration, representing 10.5% of its variation [153]. As it is not practical or financially possible to conduct sufficiently large RCTs of vitamin D supplementation in vitamin D–deficient populations, Mendelian randomization studies are likely to provide the strongest evidence on vitamin D and disease, including fractures and BMD.

## 13. Conclusions and future directions

There is mixed evidence on the effectiveness of vitamin D supplementation for the prevention of bone loss and minimal trauma fractures in postmenopausal women and older men. There is likely to be a benefit on primary fracture prevention for those with the highest background fracture risk, including those patients who have inadequate serum levels of 25(OH)D and institutionalized patients, but only when combined with calcium supplements. There is little evidence that vitamin D alone can prevent fractures, and no evidence that the combination of calcium and vitamin D, or either alone, can prevent fractures in patients with preexisting minimal trauma fractures. Recent large trials of vitamin D supplementation with multiple health outcomes, including fractures, contained only small proportions of participants with vitamin D deficiency or insufficiency. Because trials in large populations with vitamin D deficiency are not feasible because of ethical issues and expense, consideration should be given to pooling individual patient data from these studies to examine the effects of vitamin D supplementation on fractures in those with vitamin D deficiency.

The mechanisms occurring in muscle and bone, whereby a high single dose of vitamin D may result in the unexpected finding of falls and fractures, respectively, need to be determined. In addition, the role of recently identified genetic variants in explaining the high variability of clinical responses of serum 25(OH)D concentrations to either UVB exposure or to vitamin D supplementation needs to be elucidated using Mendelian randomization studies. Other less common genetic variants that may also affect serum 25(OH)D levels need to be identified to determine individualized doses of vitamin D supplements to attain optimal target levels of serum 25(OH)D. Finally, the links between the vitamin D and sex steroid axes in regulating age-related bone loss in both sexes need to be elucidated.

## 14. Summary points

- Vitamin D is a threshold nutrient, with a threshold in vitamin D status below which disease risk increases and vitamin D supplementation is beneficial.
- Recent RCTs have shown vitamin D supplementation alone increases BMD in individuals with baseline serum 25(OH)D concentrations <30 nmol/L.
- There is also likely to be a benefit on primary fracture prevention for those with the highest background fracture risk, including those patients who are

vitamin D deficient and institutionalized patients, but only when combined with calcium.

- Daily or monthly doses of vitamin D alone do not prevent fractures in community-dwelling, vitamin D-replete older adults, nor can the combination of calcium and vitamin D, or either alone, prevent fractures in patients with preexisting minimal trauma fractures.
- Mendelian randomization studies create new opportunities to examine the effects of vitamin D on bone health.

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# Adult vitamin D deficiency—fracture and fall prevention: findings from randomized controlled trials

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## OBJECTIVES

- Characterize the population of older adults that may benefit from supplemental vitamin D.
- Summarize recent evidence that vitamin D affects fall risk.
- Define the pattern of the association of circulating 25-hydroxyvitamin D (25(OH)D) levels with fall risk and estimate the 25(OH)D levels associated with minimal risk of falling.
- Summarize current evidence linking vitamin D supplementation with fracture risk in older adults and identify the dose of supplemental vitamin D that is most likely to lower fracture risk.
- Consider the potential complementary role of combined vitamin D and calcium supplementation in reducing fracture risk.

## 1. Introduction

Falls have many causes and many consequences. Leading causes, in descending order of importance, include muscle weakness, history of falls, gait deficit, balance deficit, use of assistive device, visual defect, arthritis, impaired activity of daily living, depression, cognitive

impairment, and age >80 years [1]. With aging, fall rates increase by 10% per decade (on an absolute scale) and one in three community-dwelling people over age 65 and one in two over age 80 fall each year [2]. The consequences of falls in older adults are serious. One in five falls require medical intervention, and serious injuries occur with 10%–15% of falls; for instance, 5% of falls result in a fracture and 1%–2% in a hip fracture. Moreover, over 90% of fractures occur as a result of a fall. It is well known that falls may lead to psychological trauma often described as fear of falling [3]. After their first fall, about 30% of persons develop fear of falling that results in self-restriction of activities and decreased quality of life [4]. A mitigating effect of vitamin D on fall risk was initially suspected because of the established link between severe vitamin D deficiency and proximal muscle weakness and because of evidence that vitamin D replacement improves balance. The specific role that vitamin D plays in muscle strength and balance, and athletic performance will be addressed in Chapters 29 and 38 respectively, whereas this chapter will focus on falls.

Incident fractures have enormous health and economic consequences. Fractures are estimated to increase from 2.05 to >3 million between 2005 and 2025 in US adults aged 50 years and older, with associated costs in the first year after the fracture of between \$16.9 and \$25.3 billion [5]. The older adult population is expanding, and the cost of healthcare in the United States, currently at 1.5 trillion dollars per year, is rising at an

alarming rate. In this chapter, we will review the evidence, derived largely from randomized, controlled clinical trials, linking vitamin D supplementation with risk of fracture, considering the roles of dose, dose frequency, initial and achieved 25(OH)D levels, and the presence or absence of concurrent calcium supplementation. Compatible with the Institute of Medicine (now the National Academy of Medicine) definitions [6], vitamin D insufficiency is defined by a 25(OH)D level in the range of 20–50 nmol/L (8–20 ng/mL); levels below this range are considered deficient and those above it sufficient.

## 2. Vitamin D and falls

Much of the early evidence related to whether supplemental vitamin D affects fall risk was synthesized in a 2009 metaanalysis of randomized, double-blind, placebo-controlled vitamin D intervention trials [7,8]. This metaanalysis included eight trials in 2426 individuals in whom high quality falls data were acquired prospectively, and mean age of participants was  $\geq 65$  years [9–16]. Baseline 25(OH)D levels in these trials were in the range of 25–60 nmol/L with the exception of one trial in which it was 76 nmol/L. In the eight trials, there was a significant 27% reduction in odds of falling with vitamin D treatment overall [odds ratio 0.73 (95% CI, 0.62–0.87)], a 34% reduction among trials testing doses in the range of 700–1000 IU per day [0.66 (0.53–0.82)], and no significant reduction in trials testing lower doses ranging from 200 through 600 IU per day [1.14 (0.69–1.87)] [8]. This analysis suggests that in vitamin D–insufficient older adults, supplementation with 700–1000 IU per day will lower risk of falling, whereas lower doses are ineffective.

In 2015, after the publication of this metaanalysis, two falls trials were published. A 2-year trial testing 800 IU per day of vitamin D<sub>3</sub> versus placebo found no significant benefit from supplementation in 409 community-dwelling Finnish women, aged 70–80 years [17]. The mean serum 25(OH)D level at baseline in this trial was 67.5 nmol/L, indicating that most of the women were vitamin D replete at entry into the trial. In contrast, a trial in Brazilian women with a low initial mean 25(OH)D level of 38 nmol/L found that treatment with 1000 IU per day of vitamin D<sub>3</sub> increased the serum 25(OH)D level to 69 nmol/L and significantly reduced fall risk [odds for falling in the placebo group compared with the treated group was 1.95 (95% confidence interval 1.23–3.08)] [18].

Between 2017 and 2021, the effect of oral vitamin D<sub>3</sub> on fall risk was reported in three large clinical trials (megatrials) testing doses of vitamin D > 1000 IU per day (two of the three used bolus dosing) [19–21] and in a fourth large trial testing 1000 IU per day [22]. In

the first, the vitamin D assessment (ViDA) study in New Zealand, 5110 adults aged 50–84 years were treated with an initial oral dose of 200,000 IU of vitamin D<sub>3</sub> followed by 100,000 IU per month (equivalent to 3333 IU per day) or placebo for 3.4 years. The mean serum 25(OH)D level was 63 nmol/L at baseline, and it increased to 135 nmol/L in the supplemented group. The supplementation did not alter the risk of first fall (HR 0.99 [95% CI, 0.92–1.05]). In the second megatrial, the VITAL study, treatment with 2000 IU of vitamin D<sub>3</sub> per day versus placebo for 5.3 years had no significant effect on risk of falling in 25,871 adults mean age 67.1 years [20]. In participants in the vitamin D group who had paired 25(OH)D measurements at baseline and 1 year, the serum 25(OH)D level started at 74 nmol/L and increased to 104 nmol/L. In the third megatrial, fall risk was also assessed in a survey of 16,000 randomly selected participants in the Australian D-Health Trial [21]. In this trial, men and women aged 60–84 years were randomized to treatment with 60,000 IU of vitamin D<sub>3</sub> per month or an inactive placebo. In the subset measured, intra-trial 25(OH)D levels were 77.5 in the placebo group and 114.8 nmol/L in the supplemented group. After 4.3 years of the intervention, the odds ratio for fall risk reduction was not significant (1.02 [0.95–1.10]). Falls were assessed by self-report.

The fourth megatrial, the STURDY study, was the only recent megatrial in which fall prevention was the primary endpoint [22]. This study had two phases, a 2-year dose finding phase followed by a 2-year confirmatory phase. In the dose-finding phase, participants at high risk for falling were randomly assigned to treatment with 1,000, 2,000, or 4000 IU of D<sub>3</sub> per day or 200 IU per day (control). Over 2 years of treatment, the most promising dose for fall prevention appeared to be the 1000 IU per day dose. This dose and the control dose were carried forward in the 2-year confirmatory phase in 674 adults aged 70 years and older [22]. The participants had an initial mean 25(OH)D level of 55 nmol/L that increased to 80 nmol/L in the 1000 IU arm. The confirmatory phase of this trial was null, with a hazard ratio for first fall of 0.94 [0.76–1.15].

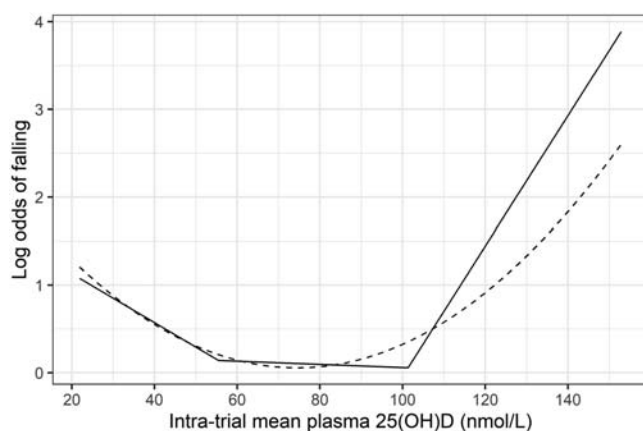
How can we reconcile the large and inconsistent body of randomized, controlled trial evidence indicating that supplemental vitamin D does and does not reduce fall risk? This question can be considered in the context of several design features of these studies.

### 2.1 Study population 25(OH)D levels

As pointed out by Morris et al. [23], a basic principle of nutrition is that most nutrients have a nonlinear association with optimal physiological function. Low nutrient levels result in poor function; there are a fairly

broad range of levels that result in optimal functioning, and high levels result in suboptimal function [23]. This pattern was in fact observed in relation to fall risk in a recent secondary analysis of the STOP/IT trial in community-dwelling men and women aged 65 years and older [24]. Participants were treated with either 700 IU of vitamin D<sub>3</sub> plus 500 mg of calcium or double placebo daily for 3 years. In an analysis of all participants combined ( $n = 410$ ), we examined the association of the cumulative mean intra-trial 25(OH)D (defined as the mean of biannual plasma 25(OH)D measurements up to and including the time of the first fall) with risk of falling. There was a statistically significant nonlinear U-shaped pattern in the association of cumulative mean 25(OH)D levels with risk of first fall (Fig. 71.1) [24]. Falls were greater in participants with intra-trial 25(OH)D levels below 50 nmol/L, were at their nadir at 25(OH)D levels in the range of 50–100 nmol/L, and were more frequent at 25(OH)D levels  $\geq 100$  nmol/L. A limitation of the analysis is that only 24 participants had intra-trial mean 25(OH)D levels  $>100$  nmol/L.

Although none of the trials published to date limited enrollment to deficient and insufficient populations, participants enrolled in the earlier trials frequently had insufficient and deficient 25(OH)D levels. In contrast, participants enrolled in the more recent trials were generally replete in vitamin D because the trials were conducted in locations in which vitamin D supplementation and food fortification had become commonplace. As a consequence, mean 25(OH)D levels at entry, ranging from 63 to 77 nmol/L in the megatrials, were consistently in the nadir region of the fall risk curve (Fig. 71.1). Thus, recent megatrials do not address the question of whether vitamin D supplementation affects fall risk in insufficient or deficient adults.



**FIGURE 71.1** Intra-trial mean plasma 25(OH)D and risk of falling from Dawson-Hughes et al. [24], with permission. Analyses, adjusted for age (half decades), sex, and calcium intake, are estimated from quadratic (dashed line) and three-piecewise logistic regression (solid line).

## 2.2 Dose size

Another feature of recent megatrials [19–21] is that they tested doses that were higher than the amounts of 700–1000 IU per day, shown earlier to reduce risk of falling in vitamin D–insufficient older adults [25]. These studies confirm that higher doses do not add value in replete individuals.

## 2.3 Safety signals

The recent megatrials, with their replete starting 25(OH)D levels and high dosing, may have placed some participants in the portion of the curve in which an adverse effect on falls is expected (Fig. 71.1). While the D-Health study overall was null for fall prevention, in the subset of 4681 participants with BMI levels  $<25$  kg/m<sup>2</sup>, supplementation with 60,000 IU of D<sub>3</sub> per month significantly *increased* risk of falling (HR 1.25 [1.09–1.43]) [21]. The achieved 25(OH)D level in this subset was not reported, but it is plausible that it was higher than the mean level achieved by participants with BMI  $\geq 25$  kg/m, because hepatic 25-hydroxylase (CYP2R1) activity is reduced in obesity [26]. Evidence that high-dose vitamin D increases fall risk has been documented in several earlier studies. Bischoff-Ferrari treated older adults at high risk for falling with 60,000 versus the standard dose of 24,000 IU of D<sub>3</sub> per month (equivalent to 2000 vs. 800 IU per day) and found significantly more falls in the higher dose group [27]. Additionally, Smith et al. tested multiple oral doses of vitamin D<sub>3</sub> ranging from 400 to 4800 IU per day and found that there were more falls in the dose groups that achieved mean 25(OH)D levels  $>103$  nmol/L [28]. In contrast, the lowest rate of falls was seen at the doses resulting in achieved 25(OH)D levels in the range of 88–103 nmol/L [28].

## 2.4 Dose frequency

Another consideration is whether “bolus” dosing has the same physiologic effect as an equivalent dose given daily. This issue came into focus with the publication by Sanders et al. who administered a single oral dose of 500,000 IU of vitamin D<sub>3</sub> or placebo annually for 4 years to 2256 women aged 70 years and older [29]. To the dismay of many, there were significantly more falls (and fractures) in the vitamin D group than in the placebo group (HR 1.16, 95% CI, 1.06–1.28). It is difficult to determine whether this untoward outcome resulted from the infrequent dosing, the magnitude of the infrequent dose administered, or both.



## 2.5 Potential physiologic basis for adverse effect of bolus doses and high daily doses of vitamin D

There is a physiologic basis for suspecting that bolus dosing and high daily dosing are counterproductive. Like modest daily dosing, bolus dosing and high daily dosing of vitamin D<sub>3</sub> effectively raise the circulating 25(OH)D concentration, but bolus dosing and high daily dosing have other effects on vitamin D metabolism. As reviewed recently by Mazess [30], bolus doses activate 24-hydroxylase (CYP24A1), which promotes conversion of 25(OH)D to the inactive metabolite, 24,25(OH)<sub>2</sub>D, thus reducing the substrate for the generation of the active metabolite, 1,25(OH)<sub>2</sub>D. It appears that both intermittent high dosing *and* high daily dosing of vitamin D (specifically 2000 to 3000 IU per day producing 25(OH)D levels  $\geq 100$  nmol/L) stimulate the production of fibroblast growth factor 23 (FGF23) [31,32], a compound that activates 24-hydroxylation (CYP24A1), which increases the catabolism of 1,25(OH)<sub>2</sub>D (and also reduces its production) [33]. The expected net effect of bolus dosing and of high daily dosing is higher circulating levels of 25(OH)D but lower levels of the active metabolite, 1,25(OH)<sub>2</sub>D. It is plausible that bolus dosing and high daily dosing with vitamin D activate a counter-regulatory endocrine response to prevent vitamin D toxicity and in so doing, lower circulating 1,25(OH)<sub>2</sub>D levels. The resulting lower 1,25(OH)<sub>2</sub>D levels may contribute to the observed increased risk of falling. Additional evidence is needed to support the clinical relevance of these proposed alterations in vitamin D metabolism.

## 3. Vitamin D and fracture risk

### 3.1 Bone mineral density

Low bone mineral density (BMD) is an established risk factor for fracture, and vitamin D supplementation has been shown in several studies to affect BMD in insufficient and deficient older adults. Ooms et al. treated 348 women aged 70 years and older with 400 IU of vitamin D<sub>3</sub> or placebo for 2 years [34]. In the vitamin D group, the mean serum 25(OH)D level was 27 nmol/L at baseline and 62 nmol/L on treatment. They found that supplementation significantly reduced bone loss at the femoral neck. Similarly, in the STOP IT trial, treatment with 700 IU of vitamin D<sub>3</sub> plus 500 mg of calcium daily for 3 years reduced bone loss from the spine and hip in 381 men and women aged 65 years and older [35]. Macdonald et al. examined the effects of 400 IU and 1000 IU per day of vitamin D<sub>3</sub> versus placebo on 1-year change in BMD in 265 older men and women and found that the higher dose modestly

decreased bone loss from the hip, whereas the 400 IU dose had no effect on hip BMD, when compared with placebo [36]. The study population was largely insufficient, with a mean baseline 25(OH)D level of 34 nmol/L. Two more recent trials have demonstrated that supplemental vitamin D in doses of 800 IU per day and 50,000 IU twice monthly for 1 year [37] and in a dose of 100,000 IU per month for 2 years [38] had no significant effect on BMD in vitamin D sufficient older adults with starting 25(OH)D levels of 52.5 nmol/L and 55 nmol/L, respectively. Collectively, these studies indicate that vitamin D supplementation modestly reduces bone loss in insufficient older populations, but not in those with adequate 25(OH)D levels.

### 3.2 Fractures

Vitamin D repletion is expected to be effective in lowering fracture risk in vitamin D—insufficient and vitamin D—deficient adults because it lowers risk of falling and improves BMD, but despite this, the evidence linking supplemental vitamin D to fracture risk reduction is inconsistent. Many randomized controlled trials have been performed to determine the effect of vitamin D on fracture risk. They have tested different doses, different dose frequencies, and interventions that involved vitamin D alone and vitamin D in combination with calcium supplementation. The earlier trials were conducted before the era of widespread vitamin D supplementation when substantial proportions of the general older population were insufficient. These earlier trials generally tested modest daily doses of vitamin D (up to 1000 IU per day) versus placebo on incident fracture, and they often included a calcium supplement along with the vitamin D. In contrast, recent vitamin D intervention trials have tended to test higher doses of vitamin D that were given daily or at less frequent intervals and have not included supplemental calcium. There have been as many metaanalyses as there are trials; we will examine in some detail the findings of two of these metaanalyses, an early individual participant-level metaanalysis of trials published between 1992 and 2010 [39] and a recent metaanalysis that includes trials published through 2018 [40].

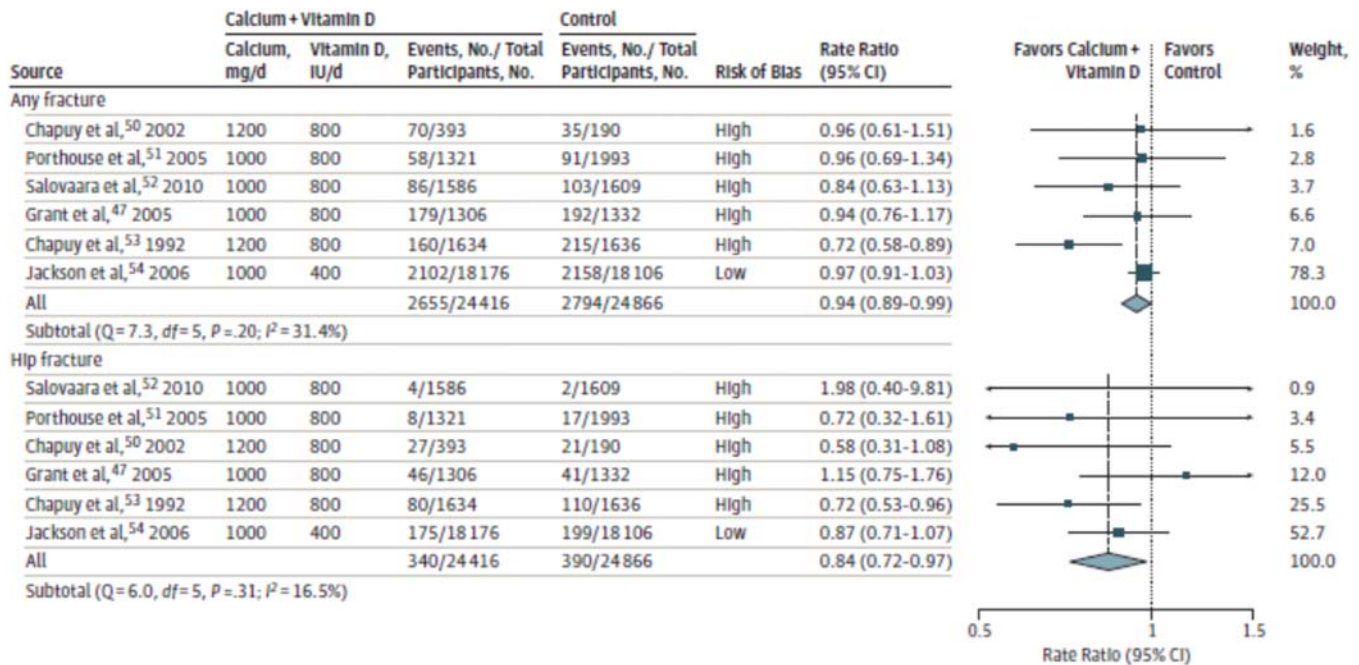
The earlier pooled analysis focused on the role of the dose administered and the actual intake of vitamin D (the product of administered dose and proportion taken) during the trial [39]. This detailed analysis was possible because individual participant-level data from the 11 included clinical trials were pooled. This analysis involved 31,022 persons, mean age 76 years, 91% of whom were women. Vitamin D supplements were given in varying amounts and at different intervals (daily, weekly, every few months). There were 1111 incident

hip fractures and 3770 non-vertebral fractures during these trials. The mean baseline 25(OH)D level was approximately 47 nmol/L, indicating that over one half of the study population was insufficient at entry into the trials. Overall, supplementation resulted in a non-significant risk reductions of 7% in non-vertebral fractures (0.93 [0.87 to 0.99]), 10% in hip fracture (hazard ratio [HR] 0.90; 95% CI, 0.80–1.01). When examined by quartiles of actual vitamin D intake during the trial (calculated as the product of dose administered X proportion consumed), there was a significant reduction in fracture risk only in the highest intake quartile. The median vitamin D intake in this quartile was 800 IU per day (range 792–2000 IU per day) [39]. The fracture risk hazard ratios in this quartile were 0.86 (95% CI, 0.76–0.96) for non-vertebral fracture and 0.70 (95% CI, 0.58–0.86) for hip fracture. This meta-analysis suggests that supplementation with 800 IU per day will lower non-vertebral fracture risk and hip fracture risk in older adults with a mean 25(OH)D level below 50 nmol/L. Notably, the majority of the included trials co-administered calcium with the vitamin D.

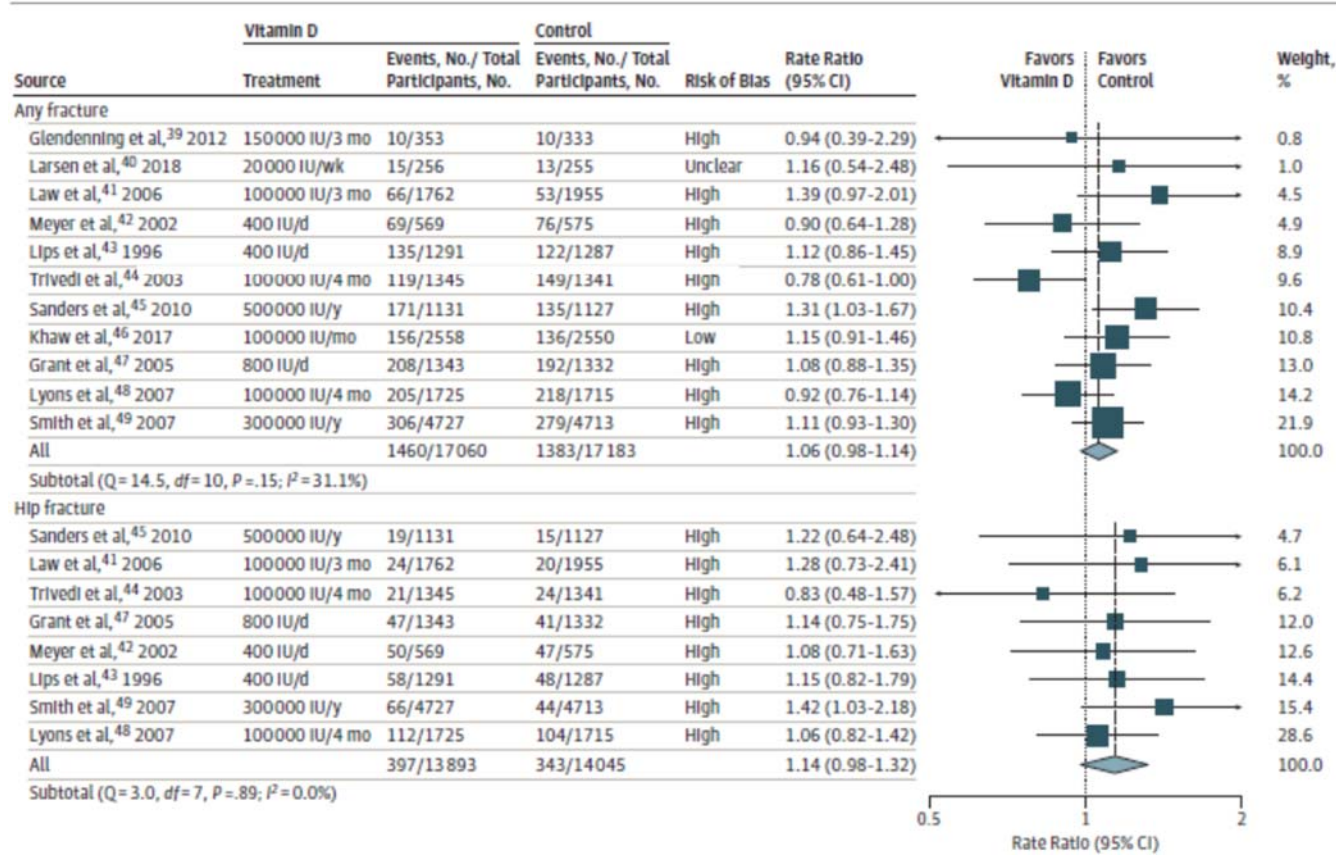
A more recent meta-analysis of randomized, placebo-controlled trials carried out by Yao et al. examined the impact of the combination of vitamin D plus calcium and vitamin D alone (vs. placebo) on fracture risk [40]. Only trials that enrolled 500 participants or more were included. Six vitamin D plus calcium trials were included in the analysis, and they involved 49,282 participants with a total of 5449 non-vertebral fractures

and 730 hip fractures. Five of the six trials tested the dose of 800 IU per day plus 1000–1200 mg per day of calcium, and the sixth trial, the Women's Health Initiative, tested 400 IU per day plus 1000 mg per day of calcium. Overall there was a 6% reduction in any non-vertebral fracture risk [0.94 (0.89–0.99)] (Fig. 71.2, upper panel) and a 16% risk reduction in hip fracture [RR 0.84 (95% CI, 0.72–0.97)] (Fig. 71.2, lower panel). Although 25(OH)D levels were not reported in the Yao metaanalysis, five of the six trials included in the vitamin D plus calcium metaanalysis reported baseline 25(OH)D levels, and they were in the range of 21–50 nmol/L [41–45].

The 11 trials in the vitamin D alone metaanalysis of Yao et al. included 34,243 participants. There were 2843 incident non-vertebral fractures and 740 hip fractures. Of these, all 11 reported non-vertebral fractures and 6 reported hip fractures. There was a lot of variation in the vitamin D doses tested and in the dose frequency. Two of the 11 trials tested a dose of 400 IU per day [46,47], and the others tested higher doses at varying intervals (one dosed weekly, one monthly, four either every 3 or 4 months, and two annually). Only one of these trials tested a dose of 800 IU per day in insufficient older adults (baseline 25(OH)D level 38 nmol/L), and it was null [43]. The overall impact of supplementation in these trials was null, with a relative risk (RR) for any fracture of 1.06 (0.98–1.14) (Fig. 71.3, upper panel). In the six trials that assessed the effect of vitamin D alone on hip fracture risk, the RR was 1.14 (0.98–1.32),



**FIGURE 71.2** Metaanalysis of randomized clinical trials of supplementation with vitamin D plus calcium or no treatment for prevention of any fracture (upper panel) or hip fracture (lower panel), from Yao et al. [40] with permission.



**FIGURE 71.3** Metaanalysis of randomized clinical trials of supplementation with vitamin D alone or no treatment for prevention of any fracture (upper panel) or hip fracture (lower panel), from Yao et al. [40] with permission.

reflecting no benefit and possibly suggesting potential harm (Fig. 71.3, lower panel). The subsequently published DO-HEALTH trial was consistent in finding no effect of 2000 IU of vitamin D per day on non-vertebral fracture risk in vitamin D–sufficient older adults [48].

4. Conclusions

In older adults who are vitamin D insufficient and deficient (defined as having 25(OH)D levels <50 nmol/L), supplementation to achieve a 25(OH)D level in the range of 50–100 nmol/L is likely to lower risk of falling. This 25(OH)D range can be achieved in most individuals through supplementation with 800 IU of vitamin D<sub>3</sub> per day. Older adults with levels of 25(OH)D in the optimal range of 25(OH)D will not reduce fall risk with supplementation. Vitamin D supplementation that achieves 25(OH)D levels greater than approximately 100 nmol/L are associated with increased risk of falling and should therefore be avoided. Intermittent high doses of vitamin D have either no effect or a deleterious effect on falling.

The physiologic basis for an increased fall risk at higher vitamin D doses is not yet established, but it may involve a counter-regulatory endocrine response to high 25(OH)D levels. It is sobering to consider that the null falls megatrials, many of which used bolus dosing, may represent a spectrum of beneficial and harmful individual responses that, when combined, appear as null effects.

In vitamin D–insufficient older adults, vitamin D in a dose of 800 IU per day together with calcium modestly reduced risk of non-vertebral fractures and, importantly, substantially reduced risk of hip fractures. Only one trial tested the dose of 800 IU per day of vitamin D alone in insufficient adults, and it was null [43]. Thus, we cannot conclude that 800 IU of vitamin D alone is effective in preventing fractures in insufficient older adults. There is now ample evidence that higher doses of vitamin D and infrequent dosing of vitamin D are not effective in lowering fracture risk. Current evidence indicates that in older adults with vitamin D insufficiency, daily supplementation with 800 IU of vitamin and an adequate intake of calcium is a rational and effective approach to reducing risk of falls and fractures.



## 5. Summary points

- Older adults most likely to benefit from supplemental vitamin D are those with circulating 25(OH)D levels in the insufficient and deficient ranges (below 50 nmol/L).
- In vitamin D insufficient older adults, supplemental vitamin D in the dose range of 700–1000 IU per day lowers risk of falls; lower doses are ineffective, and higher doses (daily or intermittent) may increase risk of falling.
- There appears to be a U-shaped association of circulating 25(OH)D levels with risk of falling in older adults. Fall risk appears to be minimal at circulating 25(OH)D levels in the range of 50–100 nmol/L and to increase progressively as levels drop below and also potentially as they rise above this range.
- Many earlier clinical trials tested the dose of 800 IU per day of vitamin D together with 1000 to 1200 mg per day of calcium in vitamin D–insufficient older adults. A metaanalysis of these trials revealed significant fracture risk reduction in non-vertebral fractures and in hip fractures. Only one trial tested 800 IU of vitamin D alone, and this trial was null. We cannot therefore conclude that vitamin D alone will reduce fracture risk.
- In conclusion, while 700–1000 IU per day of vitamin D can be expected to reduce fall risk, only 800 IU of vitamin D together with calcium has been demonstrated to reduce fracture risk in insufficient older adults. It therefore seems prudent to recommend the combination of around 800 IU of vitamin D and supplemental calcium to minimize risk of both falls and fractures in vitamin D–insufficient older adults.

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## Further reading

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# Randomized clinical trials of vitamin D and bone health

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## OBJECTIVES

- Review the results of randomized clinical trials investigating the effects of supplemental vitamin D versus placebo on bone mineral density.
- Review the results of randomized clinical trials investigating the effects of supplemental vitamin D versus placebo on incident fracture risk.
- Present the results of randomized controlled trials of the effects of supplemental vitamin D versus placebo on bone health measures, according to baseline serum 25-hydroxyvitamin D levels.
- Discuss the clinical patient population that may have skeletal benefits from vitamin D and calcium supplementation.

calcium absorption, improving mineralization of bone, reducing secondary hyperparathyroidism, and modifying bone turnover. Furthermore, extrarenal activation of 25-hydroxyvitamin D [25(OH)D] to 1,25-dihydroxy vitamin D [1,25(OH)<sub>2</sub>D] in bone has been linked to generation of osteoblast precursors [4,5]

Use of vitamin D supplements in the United States has increased markedly over the past 20 years with 36.9% of US adults aged 60 and over taking vitamin D supplements [6–8]. Although vitamin D supplements are widely used in the general population to benefit bone health, findings from randomized controlled trials (RCTs) have been inconsistent, as summarized in Table 72.1 [9,10,12–16].

## 1. Introduction: clinical and public health importance

Osteoporosis and vitamin D deficiency have been considered major public health problems (see Chapters 62, 63, 70, and 71). An estimated 53.6 million Americans have osteoporosis and/or low bone mass, which are associated with increased risk of fractures [1]. In the United States (U.S.), one in two women and one in four men aged 50 years and older will suffer an osteoporotic fracture, leading to excess morbidity and premature mortality [2,3]. As the population ages, there is an exponential rise in osteoporotic fractures [3]. Vitamin D may promote bone health by increasing intestinal

## 2. Findings from systematic reviews and metaanalyses

### 2.1 Systematic reviews and metaanalyses of supplemental vitamin D on incident fractures

Systematic reviews and metaanalyses have shown varying results of effects of supplemental vitamin D on bone mineral density (BMD) and fractures [17–24]. The 2011 US Preventive Services Task Force (USPSTF) summary of five RCTs (n = 14,583) concluded that vitamin D supplementation versus placebo was not associated with reduction in fractures [17–23]. An analysis of seven RCTs, including 68,500 older adults, in the Vitamin D Individual Patient Analysis of Randomized Trials (DIPART), also found that bolus or daily supplemental vitamin D (400 and 800 IU/day) alone did not

**TABLE 72.1** Large randomized controlled trials of supplemental vitamin D on fractures: daily and bolus dosing.

	Study	Participants	Intervention	Duration	25(OH)D levels	Key findings
Daily dosing	Lips et al. [9]	Netherlands: women and men $\geq 70$ years old, mean $\pm$ SD age $80 \pm 6$ , from both independent and residential facilities (n = 2578)	Oral vitamin D <sub>3</sub> 400 IU/day or placebo	3.5 years (median)	Baseline: 27.0 nmol/L in vitamin D <sub>3</sub> group and 26.0 nmol/L in placebo group Follow-up (3 years): 60 nmol/L in vitamin D <sub>3</sub> group and 23 nmol/L in placebo group	No effect on hip or peripheral fractures
	Bischoff-Ferrari et al. [10] DO-HEALTH	Europe: women and men $\geq 70$ years old, mean $\pm$ SD age $74.9 \pm 4.4$ (n = 2157)	Oral vitamin D <sub>3</sub> 2000 IU/day, omega-3 fatty acids 1000 mg/day, and strength-training exercise program alone and in combinations or placebo	3.0 years (median)	Baseline: $55.9 \pm 21$ nmol/L Follow up (3 years): 93.8 nmol/L in the vitamin D <sub>3</sub> groups and 60.9 nmol/L in the nonvitamin D <sub>3</sub> groups	No effect on nonvertebral fractures
	LeBoff et al. [11], VITAL	United States: women aged $\geq 55$ and men aged $\geq 50$ years old, mean $\pm$ SD age $67.1 \pm 7.1$ (n = 25,871)	Oral vitamin D <sub>3</sub> 2000 IU/day or placebo	5.3 years (median)	Baseline: $76.6 \pm 25$ nmol/L (n = 16,757) Follow up (2 years): 102.8 nmol/L in the vitamin D <sub>3</sub> group and 73.4 nmol/L in the placebo group	No effect on total, non-vertebral, or hip fractures or falls.
Bolus monthly dosing	Trivedi et al. [12]	Great Britain: women and men aged 65–85 years, mean $\pm$ SD age $74.8 \pm 4.6$ (n = 2686)	Oral vitamin D <sub>3</sub> 100,000 IU every 4 months or placebo	5 years	Follow up (4 years): $74.3 \pm 20.7$ nmol/L in the vitamin D <sub>3</sub> group, $53.4 \pm 21.1$ nmol/L in the placebo group	Reduced rate of first fracture by 22%, first fracture of hip/wrist/vertebral by 33%.
	Law et al. [13]	Great Britain: women and men in residential accommodation, mean age 85 (n = 3717)	Oral vitamin D <sub>3</sub> 100,000 IU every 3 months versus control	10 months (median)	Baseline: 59 nmol/L Follow up (1 month): 99 nmol/L Follow up (3 months): 77 nmol/L	No effect on nonvertebral fractures or falls.
	Khaw et al. 2017, Vitamin D assessment study, ViDA Trial [14]	New Zealand: women and men aged 50–84 years old, mean $\pm$ SD age $65.9 \pm 8.3$ years (n = 5110)	Oral vitamin D <sub>3</sub> 100,000 IU/month with initial oral dose of 200,000 IU or placebo	3.3 years (median)	Baseline: $63 \pm 24$ nmol/L Follow-up (6 months): $129 \pm 42$ nmol/L in vitamin D <sub>3</sub> group and $75 \pm 31$ nmol/L in placebo group	No effect on nonvertebral fractures or falls.

Bolus annual dosing	Smith et al. [15]	England: women and men $\geq 75$ years old, median age of 79.1 (n = 9440)	Intramuscular vitamin D <sub>2</sub> 300,000 IU/year or placebo	3 years	Baseline (n = 43): 141.0 $\pm$ 59.2 nmol/L Follow-up (4 months): 21% increase in the vitamin D <sub>2</sub> group (P = .15)	No effect on nonvertebral or wrist fractures. Slight increase in hip or femur fractures. No effect on falls.
	Sanders et al. [16]	Australia: community-dwelling women $\geq 70$ years old, median (IQR) age 76.0 (73.1–80.2), at high risk of hip fracture (n = 2256)	Oral vitamin D <sub>3</sub> 500,000 IU/year or placebo	3–5 years	Baseline: 49 nmol/L (median) Follow-up: 120 nmol/L at 1 month, 90 nmol/L at 3 months in the vitamin D <sub>3</sub> group	Increased rate of fractures by 26% and falls by 15%.



prevent fractures [25]. Another pooled analysis of 11 RCTs of oral vitamin D supplementation, with or without calcium, as compared with placebo or calcium alone, including 31,022 adults  $\geq 65$  years (91% females), found that there was a small 7% reduction in nonvertebral fractures and no reduction in hip fracture in those assigned to supplemental vitamin D [26]. However, in participants who were in the highest quartile of vitamin D intake (median of 800 IU/day), there was a 30% reduction in hip fracture risk and 14% reduction in nonvertebral fracture risk [26]. Of note, 8 out of 11 of these studies included coadministration of calcium. More recent systematic reviews and metaanalyses have found no benefit for supplemental vitamin D on the primary prevention of fractures, even in community-dwelling adults with low 25(OH)D levels [17–24,27–32].

The extensive review by the Institute of Medicine (IOM) committee in 2011 emphasized the need for more research from large RCTs to “test the effects of vitamin D on skeletal and nonskeletal outcomes” and “possible adverse effects where present” [32]. Since that time, a number of RCTs have been performed. In this chapter, we will review findings from large RCTs investigating the effects of supplemental vitamin D versus placebo on bone density and architecture and incident fractures in community-dwelling adults from around the world.

## 2.2 Systematic reviews and metaanalyses of supplemental vitamin D and calcium on incident fractures

In 2013, the USPSTF recommended *against* the daily supplementation with  $\leq 400$  IU of vitamin D<sub>3</sub> and  $\leq 1000$  mg of calcium for the primary prevention of fractures in community-dwelling postmenopausal women and concluded that there was insufficient evidence relative to benefits and risks of daily supplementation with  $>400$  IU of vitamin D<sub>3</sub> and  $>1000$  mg of calcium [18]. This was based heavily on the results of the Women’s Health Initiative Trial (WHI), in which daily supplemental vitamin D<sub>3</sub> 400 IU and calcium 1000 mg did not reduce hip or total fracture risk compared with placebo [33,34]. It should be noted that in addition to the interventions, 55.8% participants in the WHI took their own personal calcium and/or vitamin D supplements, as was permitted in this trial [33,34]. This RCT will be further reviewed in the following.

Since 2013, results of several, but not all, metaanalyses suggest that the coadministration of calcium and vitamin D may reduce fracture risk [20,24,28,32]. Weaver et al. performed a metaanalysis of RCTs of vitamin D plus calcium supplementation on fracture incidence

and found a 15% risk reduction of total fractures and a 30% risk reduction of hip fractures [20]. A Cochrane review found similar results with a 14% reduction in nonvertebral fractures and 16% reduction in hip fractures [32]. Finally, a 2019 metaanalysis of RCTs of combined supplementation with vitamin D and calcium found a 6% reduction in any fracture and 16% reduction in hip fractures [31]. A systematic umbrella review of metaanalyses of controlled trials found that the reduction of hip and any fracture risk with calcium and vitamin D cosupplementation is likely driven by findings in institutionalized individuals [24]. As the primary role of vitamin D is to increase absorption of calcium, adequate intakes of both vitamin D and calcium are necessary for bone health.

## 3. Current recommended intakes based on bone health goals

In 2011, after a comprehensive review, the IOM committee updated the recommended dietary allowance (RDA) of vitamin D and calcium for adult men and women with a goal of supporting bone health by optimizing calcium absorption [35]. Based on studies that found that maximal calcium absorption occurs at a serum 25(OH)D level between 30 and 50 nmol/L, the RDA for vitamin D was set at 600 IU/day for adults  $\leq 70$  years old. Using data from the winter season at high latitudes to minimize contributions from sunlight, a dose of 400 IU was predicted to result in a mean circulating 25(OH)D level of 60 nmol/L, with a lower confidence interval of 52.5 nmol/L. An RDA of 600 IU/day was selected to ensure that a level of 40–50 nmol/L is achieved in 97.5% of the population, given large interstudy variance, differences in 25(OH)D assays, and uncertainty of regression analyses.

The RDA for adults over 70 years old was set at 800 IU/day of vitamin D with a goal of reducing fracture risk [35]. At the time, and to some extent today, there was a lack of dose–response data and findings on effects of vitamin D supplementation without concurrent calcium versus placebo on fracture outcomes. Since aging is associated with decreases in intestinal calcium absorption, renal production of 1,25(OH)<sub>2</sub>D, and activation of vitamin D in skin, the IOM committee concluded a higher RDA of 800 IU/day was pertinent for this high fracture risk population, without increasing risk of adverse events.

In previous years, a number of specialty professional organizations, including the Endocrine Society, American Association of Clinical Endocrinologists, and Bone Health and Osteoporosis Foundation, have recommended a 25(OH)D level of  $\geq 75$  nmol/L for patients with osteoporosis [3,36,37]. This threshold was based

predominantly on physiological and observational studies, rather than on data from RCTs. In a small physiology study, Heaney and colleagues showed that administration of vitamin D, raising 25(OH)D levels from 50 to 87.5 nmol/L, resulted in a 65% greater gut absorption of calcium, compared with no supplemental vitamin D [38,39]. When 25(OH)D levels were below 75 nmol/L, parathyroid hormone levels have been shown to increase [40]. In the large population-based National Health and Nutrition Examination Survey III, 25(OH)D levels in the range of 22.5–94 nmol/L were positively associated with bone mineral density [41]. There are no very large RCTs of supplemental vitamin D versus placebo in patients with osteoporosis.

#### 4. RCTs of effects of vitamin D supplementation on bone density

For the general population, more recent RCTs, presented herein, support the 2011 IOM recommendations of low to moderate doses of supplemental vitamin D and a lower serum 25(OH)D goal of 50 nmol/L as sufficient. In these RCTs, high daily doses (2000 IU) of supplemental vitamin D did not reduce bone loss or fractures in midlife and older men and women with baseline 25(OH)D levels of <75 nmol/L or even <50 nmol/L, indicating that a lower serum 25(OH)D goal is sufficient for bone health in the general population [11].

##### 4.1 RCTs of supplemental vitamin D versus placebo on areal bone mineral density in the general population

An ancillary study to the large VITamin D and Omega-3 Trial (VITAL) assessed effects of daily vitamin D<sub>3</sub> (cholecalciferol 2000 IU/day) versus placebo on areal bone mineral density (aBMD) in the United States [42]. VITAL investigated effects of supplemental vitamin D<sub>3</sub> and/or omega-3 fatty acids (1 g/d) versus placebo in a 2 × 2 factorial design on the primary prevention of cancer and cardiovascular disease in US men ≥50 years old and women ≥55 years old, including 5106 black participants (n = 25,871), enrolled from all 50 states [43,44]. In a subcohort of generally healthy participants from the New England region, excluding those on osteoporosis treatments or other bone active medications (n = 771; mean age ± SD = 63.8 ± 6.1 years), supplemental vitamin D<sub>3</sub> versus placebo for 2 years did not affect changes in aBMD at the spine, femoral neck, total hip, or whole body as assessed by dual X-ray absorptiometry (DXA). With a mean baseline 25(OH)D of 69.1 nmol/L, these participants may have had vitamin

D levels sufficient for bone health prior to the start of the RCT [42]. There was no effect modification of supplemental vitamin D<sub>3</sub> versus placebo on bone density when baseline total 25(OH)D levels were stratified above and below 70 nmol/L (median), 50 nmol/L, 37 nmol/L, or 30 nmol/L. Of note, only 25 participants (3.1%) in this bone density subcohort had baseline 25(OH)D levels of <30 nmol/L, consistent with the US National Health and Nutrition Examination Survey data from 2011 to 14 reporting 2.9% of the population ≥60 years having 25(OH)D levels <30 nmol/L [45].

The Vitamin D Assessment (ViDA) trial was conducted in community-dwelling older adults in New Zealand and had a lower mean baseline 25(OH)D level of ~55 nmol/L. In this RCT, bolus dosing of supplemental vitamin D (100,000 IU/month) versus placebo in 452 participants for 2 years resulted in a slight attenuation of bone loss at the femoral neck and total hip by 0.5% but had no effect on aBMD at the lumbar spine or total body [46]. In subgroup analyses, vitamin D<sub>3</sub> supplementation prevented bone loss at the spine and femoral neck among participants with very low baseline 25(OH)D levels (≤30 nmol/L). There were no differences in changes in aBMD in participants with higher baseline 25(OH)D levels.

In the Vitamin D and Cardiovascular Risk study (VICTORY), 305 women aged 60–70 years old with baseline 25(OH)D of 33.8 nmol/L from Aberdeen, Scotland, were randomized to vitamin D 400 IU daily, vitamin D 1000 units daily, or placebo. Vitamin D supplementation of 1000 IU/day for 1 year prevented bone loss at the total hip, compared with 400 IU/day or placebo [47]. There were no differences in changes in lumbar spine BMD among the treatment groups, except in participants with baseline 25(OH)D levels of ≤30 nmol/L, in whom there was a very small benefit [48].

Other RCTs have not found a benefit of supplemental vitamin D on bone density using different regimens, even in individuals with low vitamin D levels. In an RCT in 230 postmenopausal women in Madison, Wisconsin, with baseline 25(OH)D levels of 52.5 nmol/L, supplementation with two regimens of vitamin D (bolus 50,000 IU twice per month or 800 IU/day) versus placebo did not affect aBMD [49]. In an RCT of several doses of supplemental vitamin D up to 4800 IU/day and placebo in 273 older US women with baseline total 25(OH)D levels ≤50 nmol/L, there were also no differences in percent changes in aBMD. In this RCT, changes in aBMD were not associated with baseline total 25(OH)D levels [50]. Another RCT of three different bolus doses of vitamin D<sub>3</sub> supplements (12,000 IU/month, 24,000 IU/month, or 48,000 IU/month; no placebo) in 379 older UK adults also did not find any differences in aBMD changes over 1 year, and there was no effect modification by baseline total 25(OH)D levels [51].

## 4.2 RCTs of supplemental vitamin D versus placebo on areal bone mineral density in the general population: a look at free vitamin D levels

In contrast to the studies in New Zealand and Aberdeen, results from the VITAL study did not show any benefit of supplemental vitamin D on bone density or find a baseline 25(OH)D threshold that resulted in a benefit. These inconsistencies may stem from the use of serum total 25(OH)D level as a biomarker for vitamin D status. As vitamin D is primarily bound to vitamin D-binding protein in the circulation, it may be the free 25(OH)D concentrations that predominantly exert the biological effects on bone [52–56].

At present, research studies investigating the relationship between free 25(OH)D levels and the effects of supplemental vitamin D on aBMD are limited. Small RCTs of supplemental vitamin D have not found an association between changes in aBMD and baseline-free 25(OH)D levels [50,51]. The larger VITAL study in 771 adults found that baseline-free 25(OH)D levels, measured using an ELISA assay (Future Diagnostics Solutions BV, Netherlands), may better predict improvements in aBMD than total 25(OH)D levels. Among VITAL participants with baseline-free 25(OH)D levels below the median (14.2 pmol/L), there was a small benefit for vitamin D supplementation versus placebo on changes in aBMD at the spine (0.75% vs. 0.00%,  $P = .04$ ) and total hip (−0.42% vs. −0.98%,  $P = .04$ ) [42]. Whether baseline-free 25(OH)D levels identify individuals more likely to benefit from vitamin D supplementation on fracture outcomes warrants further study.

## 4.3 RCTs of supplemental vitamin D versus placebo on areal bone mineral density in high-risk populations

A larger benefit may be found in those at high fracture risk, compared with the general adult population. In an RCT performed in the Netherlands in 348 elderly women at high fracture risk (mean age 80 years; baseline 25(OH)D of ~26.0 nmol/L), 400 IU/d of vitamin D<sub>3</sub>, compared with placebo, resulted in an increase in femoral neck aBMD by 1.9% over 2 years [57]. The New Dietary Strategies Addressing the Specific Needs of the Elderly Population for Healthy Aging in Europe (NU-AGE) study, however, found that vitamin D<sub>3</sub> supplementation (400 IU/day), along with a Mediterranean diet, for 1 year had no effect on aBMD in older men and women but, in exploratory analyses, attenuated femoral neck bone loss in those with osteoporosis [58]. In a study in 150

elderly UK women who had hip fractures, vitamin D<sub>3</sub> supplementation, administered as a single bolus injection of 300,000 IU of vitamin D<sub>2</sub> (with or without 1 g/day of elemental calcium) or vitamin D<sub>3</sub> 800 IU/day with 1 g/day of elemental calcium, versus placebo also resulted in 1-year improvements in aBMD at the femoral neck by 2.7% and at the total hip by 3.5% [59].

## 4.4 RCTs of supplemental vitamin D versus placebo on volumetric bone mineral density

While areal BMD by DXA is clinically used for diagnosis of osteoporosis and fracture risk predication, there are limitations including measurement variations due to bone size, degenerative changes, increased body fat, and marrow fat [60–62]. Peripheral quantitative computed tomography (pQCT) can measure volumetric BMD that is independent of bone size and separately assesses cortical and trabecular components of bone. In VITAL, vitamin D<sub>3</sub> supplementation, compared with placebo, did not affect total, trabecular, or cortical vBMD, cortical thickness, or bone strength measures at the radius or tibia [42]. An RCT from Calgary, Canada, found that high doses (4000 IU/day or 10,000 IU/day) versus a low dose of vitamin D (400 IU/day) for 3 years in 311 healthy adults without osteoporosis resulted in loss of vBMD at the radius and tibia; this was not a placebo-controlled trial. This study raised concerns that high doses of supplemental vitamin D may adversely affect bone structure [63].

## 5. RCTs of effects of vitamin D supplementation on fracture risk

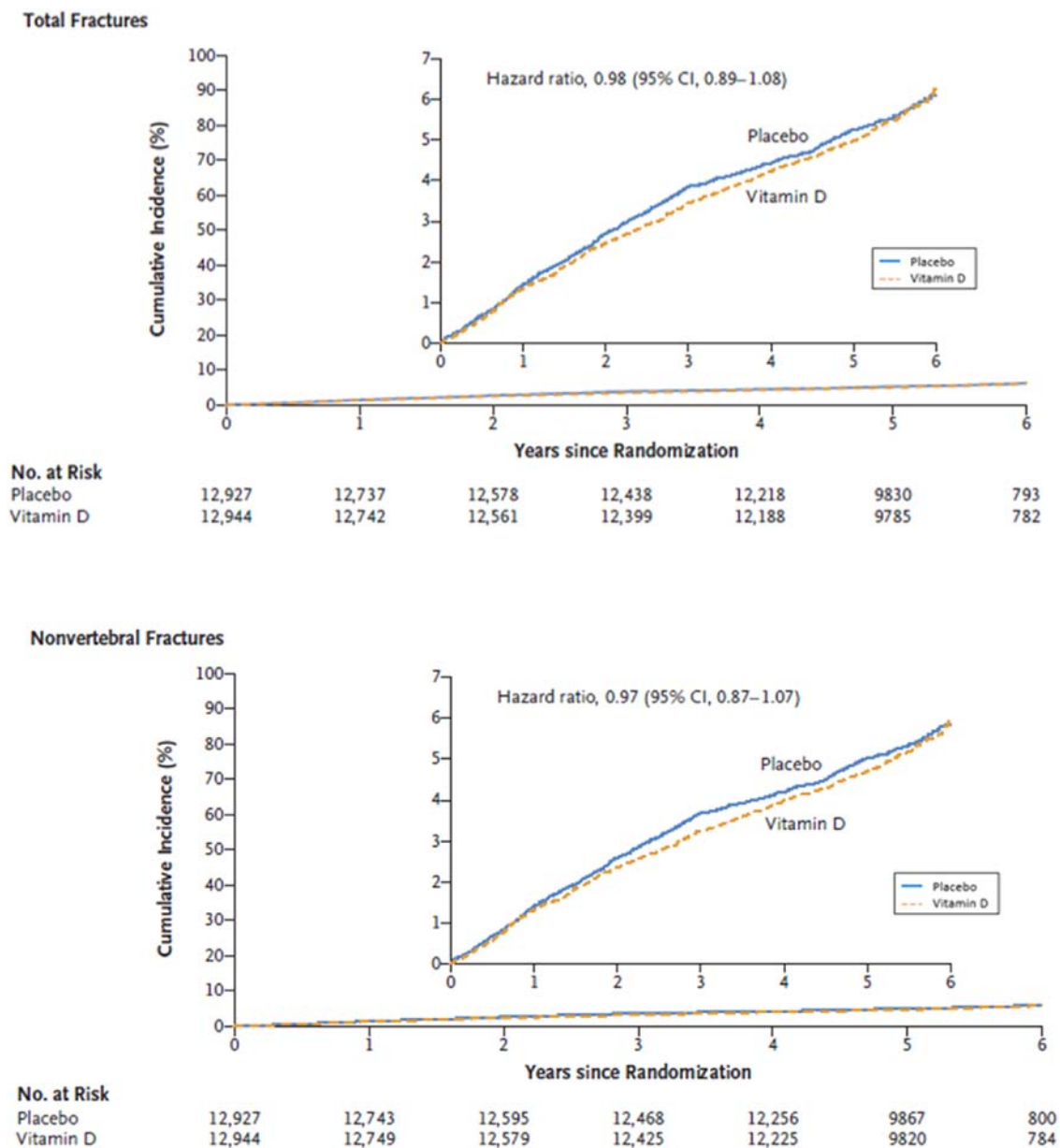
### 5.1 RCTs of supplemental vitamin D versus placebo on incident fractures in the general population

RCTs investigating effects of supplemental vitamin D on fracture risk have mostly shown no benefit, a few with benefit, and even some demonstrating potential harm [9–16]. These inconsistent findings may be due to several factors including use of bolus dosing [12,14], coadministration of vitamin D with calcium supplements [33,64], short study duration, small sample sizes, and/or differences in fracture risk of participants [10]. These variables also make metaanalyses more difficult to interpret. Table 72.1 summarizes many of these RCTs.

More recent RCTs have tested higher daily doses ( $\geq 2000$  IU/day) of vitamin D<sub>3</sub> supplementation alone (without coadministration with calcium) and have not

found a benefit for fracture outcomes. VITAL is the largest study of supplemental vitamin D<sub>3</sub> versus placebo on incident fracture risk. In VITAL, daily supplementation of vitamin D<sub>3</sub> (2000 IU/day) versus placebo did not reduce the risk of incident total (HR, 0.98; 95% CI, 0.89–1.08), nonvertebral (HR, 0.97; 95% CI, 0.87–1.07), and hip (HR, 1.01; 95% CI, 0.70–1.47) fractures in 25,871 US men aged  $\geq 50$  and women aged  $\geq 55$  years (mean  $\pm$  SD age  $67.1 \pm 7.1$ ) with a median follow-up of 5.3 years [11] (Fig. 72.1). When skull/facial, digits, peri-prosthetic, and pathological fractures were excluded, supplemental vitamin D still had no significant effect

on all outcomes. Fractures were centrally adjudicated by medical record review. Total 25(OH)D levels were measured by liquid chromatography tandem mass spectrometry (Quest Diagnostics, CA) and calibrated to Centers for Disease Control and Prevention (CDC) standards. In the active vitamin D group, mean 25(OH)D levels increased from 72.9 nmol/L to 102.8 nmol/L ( $P < .001$ ,  $n = 1347$ ) after 2 years with no significant changes in the placebo group. In exploratory analyses, there was no effect modification by age, sex, race/ethnicity, or baseline 25(OH)D levels ( $<30$ ,  $<50$ ,  $<75$ , or  $\geq 125$  nmol/L). There was limited



**FIGURE 72.1** Incident fractures in the vitamin D and placebo groups in 25,871 VITAL participants. Participants were followed for a median of 5.3 years. Incident fractures were centrally adjudicated by medical record review. Intention-to-treat analyses were performed using Cox regression models, controlled for age, sex, race, and n-3 fatty acid intervention. The insets display these same data with an enlarged y axis [11].



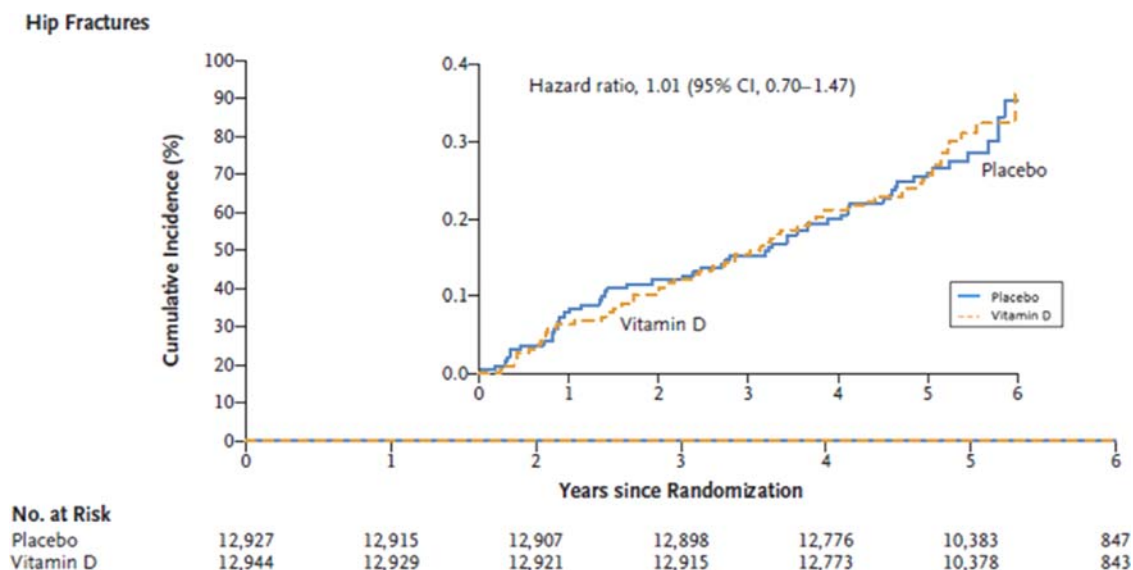


FIGURE 72.1 Cont'd

power to test the effects of supplemental vitamin D on fractures among participants with 25(OH)D levels  $<30$  nmol/L and low power in participants with history of fragility fracture or on osteoporosis treatments at baseline. Results did not change in a sensitivity analysis for adherence, and in exploratory analyses, no latency effects were observed by excluding the first couple of years of the intervention. Subgroup and post hoc analyses in VITAL were not able to identify any cohort of participants for whom supplemental vitamin D had a fracture benefit. Overall, these results only apply to healthy adults not preselected for osteoporosis, severe vitamin D deficiency, or gastric and intestinal conditions that may interfere with vitamin D absorption. This study calls into question the need for vitamin D supplementation for bone health in the general population, and small-to-moderate amounts of vitamin D through diet and/or sunlight may be sufficient in the United States.

The DO-HEALTH study investigated supplemental vitamin D of 2000 IU/day alone or in combination with omega-3 fatty acids (1000 mg/day) and a strength-training exercise program on nonvertebral fractures in 2157 European adults  $\geq 70$  year old (mean age  $74.9 \pm 4.4$ ). After a median follow-up of 3 years, there was also no effect of vitamin D supplementation on nonvertebral fracture risk in this older European cohort [10].

There has been increasing concern that very high levels of 25(OH)D may actually increase fracture risk, particularly among frail older adults, as supported by

RCTs that administered massive boluses of vitamin D supplementation. An annual oral dose of 500,000 IU of cholecalciferol in community-dwelling women 70 years or older resulted in an increased risk of fractures (RR, 1.26; 95% CI, 1.00–1.59) and an increased risk of falls (RR, 1.15; 95% CI, 1.02–1.30), particularly within 3 months of dosing [16]. Another study in men and women 75 years and older found that an annual intramuscular injection of 300,000 IU of ergocalciferol versus placebo resulted in a 49% increase in femoral fractures [15]. Of note, the baseline 25(OH)D level of this study was unusually high at 141.3 nmol/L. Similar to other endocrine feedback loops, high doses of vitamin D and 25(OH)D levels may become counterproductive by stimulating 24-hydroxylase to inactivate 1,25(OH)<sub>2</sub>D and increasing fibroblast growth factor-23 levels, which, in turn, may suppress 1 $\alpha$ -hydroxylase and downregulate activation of vitamin D [65]. Thus, the modulatory effects of vitamin D boluses may be reduced for weeks. The role of free 25(OH)D levels has not been explored in these RCTs of supplemental vitamin D versus placebo on fracture outcomes.

## 5.2 RCTs of supplemental vitamin D versus placebo on fractures in high-risk populations

There has been interest in assessing whether supplemental vitamin D versus placebo may have benefits on fracture outcomes in those with low baseline 25(OH)D

levels. As RCTs investigating the effects of supplemental vitamin D on fractures need to be large, it is difficult, and likely unethical, to select a study population with very low vitamin D levels. In a few RCTs with low baseline 25(OH)D levels, including the DO-HEALTH Trial, vitamin D supplementation did not reduce fracture risk. The ViDA study in New Zealand investigated the effects of vitamin D<sub>3</sub> 100,000 IU/month in 5110 women and men aged 50–84 years with a mean baseline 25(OH)D level of 63 nmol/L and also did not find effects on nonvertebral fractures or falls over 3.3 years [14]. Vitamin D<sub>3</sub> supplementation also did not reduce fractures in ViDA participants with baseline 25(OH)D levels <50 nmol/L (n = 1270). Similarly, in subgroup analysis of the large VITAL trial, 2161 participants had baseline 25(OH)D levels of <50 nmol/L, but no effect of supplemental vitamin D was seen in this subgroup. However, Trivedi et al. found that oral vitamin D<sub>3</sub> 100,000 IU every 4 months with 5 years of follow-up resulted in a reduction in the rate of first fracture by 22% (95% CI, 0.61–0.99) and first fracture of hip/wrist/vertebra by 33% (95% CI, 0.48–0.93) in women and men aged 65–85 years [12]. In this RCT, the 4-year follow-up 25(OH)D level was  $52.5 \pm 21.3$  nmol/L in the placebo group and  $75 \pm 20.8$  nmol/L in the vitamin D<sub>3</sub> treatment group [12].

Some RCTs have tried to target populations at higher fracture risk by older age or those living in residential communities, and these results have been disappointing. Sanders et al. specifically studied older women ( $\geq 70$  years old) with high hip fracture risk, based on history of maternal hip fracture, past fracture, or recurrent falls [16]. However, the use of annual oral dose of 500,000 IU of cholecalciferol, as mentioned before, resulted in more fractures. Law et al. investigated the use of oral vitamin D<sub>3</sub> at 100,000 IU every 3 months in elderly women and men in residential facilities (mean age 85 years old) and found no effect on nonvertebral fractures or falls after only 10 months [13]. In a Dutch study, supplemental vitamin D of 400 IU/day versus placebo in elderly adults (mean  $\pm$  SD age  $80 \pm 6$ ) from both independent and residential facilities also had no effect on hip or peripheral fractures over 3.5 years of follow-up [9]. Overall, RCTs have not suggested a strong role for supplemental vitamin D alone, without coadministration of calcium, on fracture reduction, even in high-risk populations.

### 5.3 RCTs of the effects of supplemental calcium and vitamin D on risk of fractures

The RCTs of vitamin D supplements with calcium on fracture risk have also been inconsistent [33,34,66–71]. In the Women's Health Initiative, supplemental calcium

(1000 mg/d) plus vitamin D (400 IU/d) in 36,282 postmenopausal women was not associated with a significant reduction in hip fracture risk. Subgroup analyses indicated that in women who adhered to the combined calcium and vitamin D intervention or did not take calcium and vitamin D supplements at baseline, hip fracture risk was reduced by 29% and 38%, respectively [33,34]. In contrast, in a subcohort (n = 3432) of the Osteoporosis Risk Factor and Prevention (OSTPRE) Study, an RCT of daily vitamin D (800 IU/d) and calcium (1000 mg/d) versus placebo in postmenopausal women with 3 years of follow-up did not find any effect of calcium and vitamin D supplementation on fracture incidence [67]. In VITAL, in a post hoc analysis, there was no effect modification of supplemental vitamin D versus placebo on fracture incidence in the 20% of the participants (n = 5166) who took calcium supplements ( $\leq 1200$  mg/d) at baseline or those adherent to the vitamin D intervention. Findings from these studies suggest that calcium and vitamin D supplementation do not reduce fracture risk in the general population (Table 72.2).

## 6. Implications for clinical practice

Vitamin D plays an important role in absorption of calcium, and extremely low levels of vitamin D are associated with rickets in children and osteomalacia in adults. Results from the recent large placebo-controlled RCTs indicate that in healthy midlife and older adults, high doses of vitamin D (2000 IU/day) do not provide an added benefit for fracture risk. In the United States, there may be sufficient vitamin D for bone health through diet and incidental sun exposure; the baseline 25(OH)D levels in VITAL may reflect decades of fortification of milk, some cereals, and other sources of vitamin D.

For individuals with high fracture risk with known osteoporosis or low bone density, it is reasonable and safe to treat patients with vitamin D supplements along with adequate calcium intake. However, pharmacologic therapies, including alendronate, zoledronate, denosumab, teriparatide, abaloparatide, and romosozumab, reduce fracture risk by 40%–85% and are far more effective than vitamin D and calcium supplementation alone [72–74]. Furthermore, many of these large RCTs included calcium and vitamin D supplementation in both the pharmacologic osteoporosis treatment and placebo arms. Adequate calcium and vitamin D intakes are also necessary to prevent complications of hypocalcemia with strong antiresorptive medications, particularly zoledronate and denosumab. In addition to those with osteoporosis, vitamin D supplementation is reasonable

**TABLE 72.2** Large randomized controlled trials of supplemental vitamin D and calcium on fractures.

Study	Participants	Intervention	Duration	25(OH)D levels	Key findings
Chapuy et al. [64]	Women in nursing homes or apartments for elderly, mean age $84 \pm 6$ years ( $n = 3270$ )	1200 mg of calcium and 800 IU of vitamin D <sub>3</sub> daily	18 months	Baseline: $39.9 \pm 27.5$ nmol/L in vitamin D <sub>3</sub> -calcium treatment group, $32.4 \pm 22.5$ nmol/L in placebo group Follow-up (18 months): $104.8 \pm 22.5$ nmol/L in vitamin D <sub>3</sub> -calcium treatment group, $27.5 \pm 17.5$ nmol/L in placebo group	Reduced risk of hip fractures by 43%, nonvertebral fractures by 32%.
Chapuy et al. [68]	Institutionalized women, mean age $85.2 \pm 7.1$ ( $n = 583$ )	1200 mg of calcium and 800 IU vitamin D <sub>3</sub> daily or placebo	2 years	Baseline: $21.2 \pm 13.2$ nmol/L in the intervention group, $22.7 \pm 17.2$ nmol/L in the placebo group	No effect on hip fracture.
Larsen et al. [69]	Northern Europe: Community-dwelling adults $\geq 66$ years old, median age 74 (range, 65–103) ( $n = 9605$ )	Oral calcium 1000 mg and vitamin D <sub>3</sub> 400 IU daily or control	3 years	Baseline: $37 \pm 19$ nmol/L in the intervention group, $33 \pm 19$ nmol/L in the control group Follow-up: $47 \pm 20$ nmol/L in the intervention group, $38 \pm 18$ nmol/L in the control group at 24 months	Reduced major osteoporotic fracture incidence rate by 16%.
Grant et al. [70], RECORD	United Kingdom: women and men $\geq 70$ years old, mean age of $77 \pm 6$ , with history of fracture ( $n = 5292$ )	Oral calcium 500 mg and vitamin D <sub>3</sub> 400 IU twice daily (800 IU/d), or placebo	3 years	Baseline: $37.9 \pm 16.2$ nmol/L Follow-up (1 year): Rose by $24.0 \pm 17.2$ nmol/L in combination treatment group, rose by $7.7 \pm 18.0$ nmol/L in placebo group	No effect on low-trauma, total or hip fractures.
Porthouse et al. [71]	England: women $\geq 70$ years old, mean age $77 \pm 5$ , with $\geq 1$ risk factors for hip fracture ( $n = 3314$ )	Oral calcium 500 mg and vitamin D <sub>3</sub> 400 IU twice daily (total 800 IU/d) or control	25 months (median)		No effect on hip or clinical fractures.
Jackson et al. [33], Prentice et al. [34], Women's health Initiative [33,34]	United States: women aged 50–79, mean $\pm$ SD age $62.4 \pm 7.0$ ( $n = 36,282$ )	Oral calcium 500 mg with vitamin D <sub>3</sub> 200 IU twice daily (400 IU/day) or placebo	7.0 years (mean)	Baseline: $46.0 \pm 22.6$ nmol/L among participants with previous hip fracture (in nested case-control assessment), $48.4 \pm 23.5$ nmol/L in control group Follow-up: 28% higher in active calcium + vitamin D <sub>3</sub> group compared with placebo group	No effect on hip, clinical vertebral, wrist, or total fractures. Reduced risk of hip fracture by 29% in adherent participants. Reduced risk of hip fracture by 38% in participants not on calcium and vitamin D <sub>3</sub> supplements at baseline ( $n = 15,302$ ).
Salovaara et al. [67], OSTPRE	Finland: women aged 65–71 years, mean $\pm$ SD age $67.4 \pm 1.9$ ( $n = 3432$ )	Oral calcium 500 mg and vitamin D <sub>3</sub> 400 IU twice daily (800 IU/d) or control	3 years (mean)	Baseline: $50.0 \pm 18.7$ nmol/L (mean) in the intervention group, $49.1 \pm 17.7$ nmol/L in the control group Follow-up: $74.6 \pm 21.9$ nmol/L in the intervention group, $55.9 \pm 21.9$ nmol/L in the control group	No effect on total, nonvertebral, clinical vertebral, osteoporotic, or wrist fractures.

in patients with very low 25(OH)D levels, limited sun exposure, and malabsorption or related gastrointestinal disorders.

## 7. Conclusion

Recommendations for vitamin D for the promotion of bone health and prevention of fractures continue to be studied and debated. Recent findings from large RCTs have challenged the long-standing notion that high doses of vitamin D reduce fractures in the general population. Supplemental vitamin D (2000 IU/d), compared with placebo, did not reduce fracture risk in 25,871 healthy middle-aged and older US adults over a median of 5.3 years of follow-up in VITAL [11]. Smaller doses of vitamin D may be sufficient for bone health. Furthermore, RCTs have had more difficulty finding therapeutic thresholds. The large VITAL study, which measured baseline 25(OH)D levels in 16,757 participants, did not show a benefit of high-dose supplemental vitamin D (2000 IU/d) on fracture reduction at clinically relevant thresholds. Current thresholds for bone are not fully evidence-based and may need to be reevaluated in the context of these data.

For patients with osteoporosis at high risk of fractures, pharmacological therapies can markedly reduce fracture risk. The findings in the VITAL study should not be extrapolated to individuals with low bone mass or osteoporosis. The statistical power in VITAL for effects of supplemental vitamin D versus placebo on incident fractures in the participants who had fragility fractures or were on osteoporosis medications at baseline was low. Supplementation with vitamin D and calcium in patients with low bone mass, osteoporosis, or on osteoporosis medications is safe, and we continue to recommend this for these patients.

Overall, widespread screening of serum 25(OH)D levels in generally healthy midlife and older adults should be curtailed as it is likely unnecessary for bone health outcomes. Measurement of 25(OH)D levels continues to be useful in patients with low bone mass or osteoporosis in the evaluation of secondary causes of osteoporosis such as hyperparathyroidism or malabsorption. Other studies in the large VITAL cohort have shown a few select patient populations who may benefit from vitamin D supplementation. Although supplemental vitamin D (2000 IU/d) did not decrease cancer incidence or a composite of cardiovascular outcomes in the overall VITAL cohort, in a subgroup analysis, it did decrease cancer mortality by 25% after a latency period [44,75] and autoimmune diseases by 22% [76]. Ongoing studies are investigating effects of supplemental vitamin D on infections, lung disease, and COVID among others.

## 8. Summary points

- Although vitamin D supplements are widely used in the general population to benefit bone health, findings from RCTs have been inconsistent.
- Recent large RCTs have generally found no benefits of high daily doses of supplemental vitamin D (without coadministration of calcium) on areal bone mineral density.
- Recent RCTs have also not supported the use of supplemental vitamin D to reduce incident fractures in generally healthy, community-dwelling adults in the United States and globally.
- These findings do not apply to adults with severe vitamin D deficiency, low bone density or osteoporosis, or elders living in residential communities.

The VITAL ancillary studies, *Effects of Supplemental Vitamin D on Bone Health Outcomes in Women and Men in the VITamin D and Omega-3 Trial (VITAL)*, *Supplemental Vitamin D and Incident Fractures in Midlife and Older Adults*, and *The clinician's guide to prevention and treatment of osteoporosis*, are supported by grants (R01 AR060574, R01 AR070854, and R01 AR059775, to Dr. LeBoff) from the National Institute of Arthritis and Musculoskeletal and Skin Diseases. The VITAL parent trial is supported by grants (U01 CA138962, R01 CA138962, and R01AT011729, to Drs. Manson and Buring) from the National Cancer Institute, the National Heart, Lung, and Blood Institute, the Office of Dietary Supplements, the National Institute of Neurological Disorders and Stroke, and the National Center for Complementary and Integrative Health.

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# Calcifediol as a therapeutic

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## OBJECTIVES

- To highlight the recent, novel uses of calcifediol (25-hydroxyvitamin D<sub>3</sub>) as an oral therapeutic agent to safely and effectively raise serum levels of 25-hydroxyvitamin D and, thereby, treat (i) secondary hyperparathyroidism in chronic kidney disease complicated by vitamin D insufficiency and (ii) respiratory microbial diseases including COVID-19.

## 1. Introduction

Calcifediol or 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) is at the apex of the vitamin D metabolic pathway. It is the major circulating form of vitamin D and substrate for the two principal metabolites of vitamin D<sub>3</sub>, namely 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub> or calcitriol) and 24,25-dihydroxyvitamin D<sub>3</sub> (24,25(OH)<sub>2</sub>D<sub>3</sub>), but is infrequently considered for its therapeutic potential. Here, we will consider the recent data supporting the use of calcifediol and, in particular, extended-release calcifediol (ERC) in treating: (1) vitamin D insufficiency (VDI), defined by a total serum total 25-hydroxyvitamin D (25(OH)D) level of <20 ng/mL (50 nmol/L) or <30 ng/mL (75 nmol/L); (2) secondary hyperparathyroidism (SHPT), especially in the setting of chronic kidney disease (CKD); and (3) microbial infections associated with deficient innate and adaptive immune responses to the offending microbe. The mechanism of action of

calcifediol revolves around both traditional endocrine actions of calcitriol and nontraditional production of intracrine calcitriol.

As displayed in Table 73.1, traditional endocrine and nontraditional intracrine mechanisms of action of calcifediol share a common enzymatic target (25-hydroxyvitamin D-1 $\alpha$ -hydroxylase or CYP27B1), active metabolite (calcitriol), and tissue-based receptor (vitamin D receptor or VDR), but differ in most other respects. This includes the cell types that harbor CYP27B1. The endocrine-acting CYP27B1 is concentrated in epithelial cells of the proximal renal tubule [1,2], while the intracrine-acting CYP27B1 is expressed outside the kidney, for example in parathyroid cells and in activated cells of the innate immune system ([3]; see Chapter 9). The preferred substrates for the renal CYP27B1 are the two most abundant, 25-hydroxylated, circulating metabolites of vitamin D. The first is 25(OH)D<sub>3</sub> which is largely synthesized in the liver by the CYP2R1 [4]. The second is 24,25(OH)<sub>2</sub>D<sub>3</sub>, the product of the CYP24A1, the vitamin D-specific catabolic enzyme [5]. Hydroxylation at the C-24 position initiates side chain cleavage of the molecule and its eventual catabolism to nonbiologically forms (reviewed in Jones et al. [6]; see Chapter 5).

The CYP24A1 enzyme is highly concentrated on the inner mitochondrial membrane of cells of the proximal renal tubule alongside CYP27B1, where its expression is more robust than that of the CYP27B1 (see Chapters 5 and 8). CYP24A1 is not as substrate limited or tightly regulated as CYP27B1 [6]. Consequently, in the disease free state, CYP24A1 can easily “out compete” CYP27B1 for the same substrates, leading to their catabolic deactivation. CYP24A1 is widely distributed among almost all tissues and cells of the body that express the VDR.



**TABLE 73.1** The function of the 25(OH)D<sub>3</sub> (calcifediol)/1,25(OH)<sub>2</sub>D<sub>3</sub> (calcitriol) axis in its traditional endocrine action compared to its non-traditional intracrine action.

Function	Endocrine action	Intracrine action
Cell	Proximal renal tubular epithelial cell	Target cell (e.g., macrophage)
Enzyme	CYP27B1	CYP27B1
Substrate (optimal range)	25(OH)D <sub>3</sub> (50–75 nM)	25(OH) <sub>3</sub> D (125–250 nM)
Mode of 25(OH)D <sub>3</sub> delivery	Megalin-bound DBP	Free 25(OH)D <sub>3</sub>
Source of 25(OH)D <sub>3</sub>	Urine	Serum
Substrate concentrated	Yes	No
Competition for substrate	CYP24A1 (robust)	Alternatively spliced CYP24A1
Enzymatic products	1,24,25(OH) <sub>3</sub> D <sub>3</sub> > 1,25(OH) <sub>2</sub> D <sub>3</sub>	1,25(OH) <sub>2</sub> D <sub>3</sub> > 1,24,25(OH) <sub>3</sub> D <sub>3</sub>
Major stimulator	PTH	Tissue-specific cytokines
Other major regulator	FGF-23 (inhibitor)	Toll-like receptor activation
1,25(OH) <sub>2</sub> D <sub>3</sub> target	VDR	VDR
Regulation target	Calcium/Phosphate/PTH axis	Cell-specific, VDR-driven responses

Regardless of the tissue or cell, in normal physiology the *CYP24A1* is the most VDR-responsive human gene; it contains powerful 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D)-VDR-activatable enhancer elements [7]. As such, upregulation of expression of the *CYP24A1* gene by 1,25(OH)<sub>2</sub>D is considered a means of limiting the formation and export of active, VDR-interacting hormone by that cell [7]. By contrast, unactivated innate immune cells do not constitutively express the VDR or *CYP24A1* (see Chapter 94). Even after activation by antigens and/or cytokines, the 24-hydroxylating activity in such cells is limited by the production of an alternatively spliced, amino-terminally truncated, nonbiologically active isoform of the *CYP24A1* missing its N-terminal mitochondrial targeting sequence [8,9]. This is the reason why patients with macrophage proliferating, granuloma-forming diseases like sarcoidosis or tuberculosis where the *CYP27B1* is not counterbalanced by *CYP24A1* activity, are extremely sensitive to increases in the serum 25(OH)D level and subsequent development of 1,25(OH)<sub>2</sub>D-driven hypercalciuria and/or hypercalcemia [10].

Owing to the differences in cells containing *CYP27B1*, other salient differences reside between the endocrine- and intracrine-acting pathways for 25(OH)D<sub>3</sub> (Table 73.1). Prime among them is the means by which substrate 25(OH)D<sub>3</sub> or 24,25(OH)<sub>2</sub>D<sub>3</sub> gains access to the metabolic machinery in the cell bearing the *CYP27B1*. While the source of the substrate is the same, circulating 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub> bound to the serum vitamin D binding protein (DBP), and to a lesser extent albumin, the modes of substrate delivery differ. On the

traditional, endocrine action side of the equation, 25(OH)D<sub>3</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>, whether DBP- or albumin-bound, are largely transferred from the blood into the urine through glomerular filtration. DBP with the bound vitamin D metabolite is then reclaimed from the urine by high affinity binding and internalization by the transmembrane megalin/cubulin complex residing on the luminal membrane of the proximal tubular epithelial cell [11]. Once internalized, most of the carrier DBP and its metabolite cargo are recycled back into the venous circulation by a yet poorly understood mechanism. It is hypothesized that some of the internalized DBP-vitamin D metabolite complex is incorporated into an endolysosome whose acidic environment cleaves DBP from the metabolite [12]. From this point, it is uncertain how free substrate finds its way to *CYP27B1* or *CYP24A1*, residing side-by-side on the inner mitochondrial membrane. It is presumed that the relative level of free substrate and factors controlling the *CYP27B1*-hydroxylation reaction (e.g., substrate affinity) will determine the relative output of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1,24,25(OH)<sub>3</sub>D<sub>3</sub> from that cell. Glomerular filtration of DBP and albumin and their subsequent capture by the megalin-cubulin internalization machinery is also a mode of concentrating vitamin D metabolites at the luminal surface of the proximal tubular epithelial cell, promoting facilitated access to the *CYP27B1* and *CYP24A1* that reside in that cell. This ability of the kidney to concentrate filtered DBP-metabolite complexes coupled with the megalin-cubulin facilitated uptake of those complexes by the proximal tubular epithelial cell may also help explain why the renal *CYP27B1* can

efficiently produce  $1,25(\text{OH})_2\text{D}_3$  at relatively low levels of serum substrate  $25(\text{OH})\text{D}_3$  (50–75 nM) compared to the higher range (125–250 nM) required for the CYP27B1 located in nonrenal target tissues or the activated human innate immune cell. Chapter 7 includes a more detailed discussion of the role of DBP in vitamin D metabolism and function.

As further depicted in Table 73.1 and in contrast to the relatively complex means of substrate delivery to the kidney CYP27B1 or CYP24A1, the delivery of substrate  $25(\text{OH})\text{D}_3$  to the activated innate immune cell is reliant on the availability of free  $25(\text{OH})\text{D}_3$  in the inflammatory microenvironment. The mechanism by which free  $25(\text{OH})\text{D}_3$  is delivered from the serum to the CYP27B1 in innate immune cells (such as macrophage and dendritic cells) is unknown. Because of its relatively high affinity for DBP and lower free levels,  $24,25(\text{OH})_2\text{D}_3$  is thought to enter the innate immune cell much less easily and not compete with  $25(\text{OH})\text{D}_3$  as substrate for the CYP27B1. As mentioned above, the wildtype CYP24A1 gene and its product, the full length, unspliced CYP24A1, are not highly expressed in the nontraditional immune metabolic pathway. In comparison to what happens with the endocrine-acting CYP27B1 and CYP24A1 in the kidney, where the output of the two enzymes counterbalances one another, without a robust CYP24A1-hydroxylase pathway in innate immune cells, substrate  $25(\text{OH})\text{D}_3$  is preferentially converted to  $1,25(\text{OH})_2\text{D}_3$ . This imbalance leads to the preferred production and release of  $1,25(\text{OH})_2\text{D}_3$  into the local inflammatory microenvironment with the potential for spillover into the general circulation when the total body load of disease-activated,  $1,25(\text{OH})_2\text{D}_3$ -secreting innate immune cells is high [13]. In this case, an increase of circulating  $1,25(\text{OH})_2\text{D}_3$  above normal can occur, making the host susceptible to the traditional, endocrine action of the  $1,25(\text{OH})_2\text{D}_3$  with resultant disturbance of calcium homeostasis [14].

The traditional, endocrine vitamin D operating system governing the renal synthesis of  $1,25(\text{OH})_2\text{D}$  is tightly regulated by other circulating hormones that control CYP27B1 and CYP24A1 gene expression. These hormonal regulators gain access to proximal tubular epithelial cell through the “blood” (basolateral membrane) not the “urine” (luminal membrane) side of the cell. The two key regulators of the renal CYP27B1 are both peptide hormones, parathyroid hormone (PTH) and fibroblast growth factor (FGF23) (see Table 73.1 and Chapters 8 and 19); they remain blood bound as they are too large to be filtered through the glomerulus. PTH is the major stimulator, and FGF23 is the major inhibitor of the renal CYP27B1 and 1-hydroxylase activity. PTH release is stimulated by a decrease in the serum ionized calcium and  $1,25(\text{OH})_2\text{D}$  concentration [15] and FGF23 synthesis is stimulated by a decrease in the

glomerular filtration rate (GFR) in early renal failure [16]. This “yin-yang” means of control of CYP27B1 expression is the essence of the endocrine feedback on  $1\alpha$ -hydroxylase activity (see Chapters 8 and 9). It is the concerted interplay of the  $1,25(\text{OH})_2\text{D}$ -VDR-target gene interactions in the gut, bone, parathyroid gland, and the kidney that serve to provide optimal circulating calcium and phosphate levels required for normal skeletal homeostasis. The pathophysiological outcome of the failure of this servosystem to control  $1,25(\text{OH})_2\text{D}$  serum levels in the appropriate range and normal  $1,25(\text{OH})_2\text{D}$ -VDR-driven trans-regulation of the genes in these target tissues is dysregulated calcium homeostasis.

As discussed below in the section “Calcifediol treatment of secondary hyperparathyroidism associated with chronic kidney disease,” CKD is perhaps the best clinical example of failure of the traditional, endocrine-active vitamin D system associated with bone loss and extra-skeletal arterial calcification. The latter is the major cause of morbidity and mortality in CKD [17]. Owing to a decrease in GFR in early CKD, there is a decrease in tubular epithelial cell function, including an undesired, increased retention of filtered phosphate from the urine back into the blood. Although still unclear mechanistically, the impact of phosphate retention is sensed by osteocytes buried in the calcium-phosphate hydroxyapatite matrix of bone and triggers the release of FGF23 into the general circulation [18]. FGF23, acting as an endocrine hormone, counters CKD-driven renal tubular reabsorption of phosphate, reduces the activity of the CYP27B1 to synthesize  $1,25(\text{OH})_2\text{D}$  and increases the activity of the CYP24A1 to catabolize  $1,25(\text{OH})_2\text{D}$  [19]. The net result is a reduction in the circulating concentration and action of  $1,25(\text{OH})_2\text{D}$ . The consequence of these FGF23-driven events at the level of the renal tubule is an increase in phosphate and calcium excretion and a decrease in the circulating levels of phosphate and calcium for incorporation into the mineral matrix of bone. In response to what would appear to be a clinically insignificant drop in ionized calcium level, the calcium-sensing receptor (CASR) in parathyroid tissue is activated leading to an increase in PTH secretion into the general circulation, a persistent state of SHPT and a further loss in bone mineral content [20]. SHPT associated with CKD is usually amplified by the presence of  $25(\text{OH})\text{D}$  insufficiency [21,22], leading to reduced serum  $1,25(\text{OH})_2\text{D}$  and a compensatory increase in PTH secretion.

Medial calcification of arterial vessels is a frequent and morbid event in patients with CKD [17]. This phenotypic alteration in the arterial cell wall is thought to be a consequence of events central to the overall pathogenesis of bone disease in CKD [23]. These include SHPT, namely the disturbance in the circulating balance of phosphate and calcium directed by an elevated PTH level.

Superimposed on that is CKD-associated hyperphosphatemia-induced increase in the expression of the PIT-1 and PIT-2 sodium-dependent phosphate co-transporters in arterial vascular smooth muscle cells [24]. This event promotes phosphate uptake by subluminal vascular smooth muscle cells with their resultant trans-differentiation to an osteogenic phenotype capable of deposition of hydroxyapatite (Fukagawa and Kazama [25]; see Chapters 26 and 79).

By contrast (see Table 73.1), activated innate immune cells that express the VDR and CYP27B1 are resistant to circulating PTH signaling as these cells do not express substantial amounts of the receptor for this calciotropic hormone [26]. Instead, the *CYP27B1* gene in activated innate immune cells is responsive to locally-produced cytokines like with IFN- $\gamma$  [27,28] and microbial-derived pathogen-associated membrane patterns (PAMP) and damage-associated molecular patterns (DAMP) operating through expressed toll-like receptors (TLR) on the cell membrane and endoplasmic membrane, respectively, with activation of the TLR2/1 being the most potent stimulator of the innate immune cell CYP27B1 and 1,25(OH) $_2$ D production [29]. Activation of the human monocyte/macrophage also leads to upregulated expression of intracrine 1,25(OH) $_2$ D-VDR-driven antimicrobial peptide genes (e.g., the cathelicidin antimicrobial peptide gene [*CAMP*]) [29] and cytokines like IL-1 $\beta$  that amplify the innate immune response and activate the adaptive immune response to the offending microbe in an autocrine/paracrine mode of action [30,31] (see section “Calcifediol treatment of microbial disease to boost the human innate and adaptive immune responses” below).

## 2. Treatment of vitamin D insufficiency with calcifediol

### 2.1 Pathophysiology of endocrine vitamin D insufficiency (VDI)

The canonical endocrine function of vitamin D is to promote intestinal calcium and phosphate absorption in order to maintain normal mineral balance and bone health. In the 25(OH)D insufficient state, low serum 1,25(OH) $_2$ D and associated reduction in intestinal calcium absorption [32,33] lead to a subclinical decline in circulating calcium. The decreases in 1,25(OH) $_2$ D and calcium are detected by the parathyroid gland via the VDR and CASR, respectively, prompting a compensatory release of PTH. PTH helps to restore normal circulating levels of 1,25(OH) $_2$ D and calcium by engaging the PTH/PTHrP receptor in the proximal tubular epithelial cell of the kidney, leading to upregulation of the CYP27B1 which converts available substrate 25(OH)D

to 1,25(OH) $_2$ D. 1,25(OH) $_2$ D then acts in an endocrine fashion, traveling to the gut to increase intestinal calcium and phosphate absorption, and to bone to liberate calcium and phosphorus from the mineral (hydroxyapatite) phase of bone [26]. Based on this underlying physiology, the compensatory rise in PTH resulting from 25(OH)D insufficiency is referred to as SHPT. Indeed, PTH levels are higher at lower serum 25(OH)D levels [34]. However, the precise threshold for 25(OH)D below which PTH secretion increases is uncertain, with some experts proposing a 20 ng/mL threshold [35] and others proposing a 30 ng/mL threshold [36]. Sustained hyperparathyroidism is detrimental to bone, as it promotes bone resorption [37].

### 2.2 Advantages and disadvantages of parent vitamin D to restore vitamin D health

Supplementation with parent vitamin D is the most commonly prescribed approach to restore status [36]. Although parent vitamin D is widely available and inexpensive, it has questionable effectiveness for treating the clinical sequelae of vitamin D insufficiency (SHPT and osteomalacia) [38–40]. First, the mechanism of intestinal absorption of ingested vitamin D is complex and relatively inefficient. Vitamin D, either as cholecalciferol or ergocalciferol, is absorbed via carrier proteins that mediate intestinal uptake of cholesterol, subsequently packaged in chylomicrons, and delivered to the general circulation via the lymphatic system [41–43]. In individuals without gastrointestinal comorbidities (e.g., Crohn’s disease), on average 78%–85% of ingested vitamin D is absorbed [44,45] when administered in the nonfasting state. However, in the absence of food, and in patients with intestinal fat malabsorption, absorption of vitamin D is often compromised; in those who have celiac disease, pancreatic insufficiency, biliary cirrhosis, or who are postbariatric surgery, intestinal vitamin D absorption can be <50% [44–46]. A second limitation of parent vitamin D is that its conversion to 25(OH)D may be impaired if there are inactivating mutations of *CYP27B1*, in end-stage liver disease, obesity [47,48] or CKD [49,50], or if *CYP27B1* activity is altered by interfering medications [51].

### 2.3 Comparative advantages of using calcifediol to restore vitamin D health

Although less widely available, calcifediol has important advantages compared to parent vitamin D. First is its greater bioavailability. Calcifediol is more polar and less fat soluble than parent vitamin D; accordingly, its intestinal absorption does not require bile acids and chylomicron formation and can exceed 93% [45].

**TABLE 73.2** Randomized controlled trials comparing the effects of calcifediol versus vitamin D<sub>3</sub> on circulating levels.

Author Year (ref)	Study size (N) Study duration	Study interventions	Baseline (ng/mL)	Final 25(OH)D (ng/mL)
Barger-Lux [59]	N = 116 8 weeks	D <sub>3</sub> 25 mcg/day D <sub>3</sub> 250 mcg/day D <sub>3</sub> 1250 mcg/day 25D <sub>3</sub> 10 mcg/day 25D <sub>3</sub> 20 mcg/day 25D <sub>3</sub> 50 mcg/day	26.8 (for full sample)	Only delta reported: +11.4 +58.4 +257.2 +16.0 +30.4 +82.6
Cashman [53]	N = 56 10 weeks	D <sub>3</sub> 20 mcg/day 25D <sub>3</sub> 7 mcg/day 25D <sub>3</sub> 20 mcg/day	19.9 17 15.3	27.6 28.3 53.4
Bischoff-Ferrari [54]	N = 20 4 months	D <sub>3</sub> 20 mcg/day 25D <sub>3</sub> 20 mcg/day	14.2 12.3	40.0 69.5
Navarro-Valverde [55]	N = 40 12 months	D <sub>3</sub> 20 mcg/day 25D <sub>3</sub> 20 mcg/day 25D <sub>3</sub> 266 mcg/week 25D <sub>3</sub> 266 mcg/2 weeks	16.2 14.9 15.2 15.8	34.5 75.2 93.2 84.2
Vaes [56]	N = 50 24 weeks	D <sub>3</sub> 20 mcg/day 25D <sub>3</sub> 5 mcg/day 25D <sub>3</sub> 10 mcg/day 25D <sub>3</sub> 15 mcg/day	15.1 17.4 15.3 15.4	28.6 20.9 35.5 43.0
Shieh [57]	N = 35 16 weeks	D <sub>3</sub> 60 mcg/day 25D <sub>3</sub> 20 mcg/day	16.2 17.0	29.6 42.4
Perez-Castrillon [58]	N = 303 4 months	D <sub>3</sub> 24,000 IU/month 25D <sub>3</sub> 266 mcg/month	13.2 12.8	23.1 27.8

Once absorbed, it enters the circulation directly via the portal vein [43]. Consistent with this physiology, the bioavailability of vitamin D<sub>3</sub> was only 44.8% or 35.9% that of calcifediol when administered at doses of 20 µg/day or 140 µg/week, respectively, for 4 months [52]. The second advantage of calcifediol is that its efficacy is not affected by alterations in CYP2R1 activity.

Table 73.2 summarizes previously published data from randomized controlled trials (RCT) comparing the effects of calcifediol versus vitamin D<sub>3</sub> on circulating 25(OH)D levels. In all [52–58]) but one [59] of the listed studies, the mean baseline 25(OH)D level was low ( $\leq 20$  ng/mL). Tested doses of calcifediol ranged from 5 µg/day to 50 µg/day, and treatment durations spanned 2–12 months. In aggregate, when compared to vitamin D<sub>3</sub>, calcifediol increased serum 25(OH)D levels more quickly and to a greater extent [52–59]. Indeed, at doses of 10 µg/day or higher, calcifediol consistently raised 25(OH)D levels in patients with normal renal function to  $>30$  ng/mL [52–59], the level below which PTH may begin to increase [60]. In contrast, cholecalciferol, even at doses of 60 µg/day, may not reliably increase serum levels above this threshold [57]. The use of calcifediol at studied doses did not result in hypercalcemia.

In a review of RCTs, the mean calculated relative potency of calcifediol versus parent vitamin D<sub>3</sub> was 4.6. Notably, the relative potency of calcifediol differed depending on the vitamin D<sub>3</sub> dose. When compared to vitamin D<sub>3</sub> at lower doses ( $<25$  µg/day), the mean relative potency of calcifediol was 3.2; but, when compared to vitamin D<sub>3</sub> at higher doses ( $>50$  µg/day), the relative potency was 8.0. The authors speculated that the CYP2R1 enzyme becomes saturated more quickly at higher doses of vitamin D<sub>3</sub>, limiting its ability to generate calcifediol [51].

## 2.4 Suppression of parathyroid hormone (PTH) secretion

Although the primary objective of the above studies was to compare the effects of calcifediol versus vitamin D<sub>3</sub> on circulating 25(OH)D levels, some investigators also explored their comparative effects on circulating PTH, with some suggesting superior (or trends toward superior) PTH suppression with calcifediol [53–56]. For example, Cashman et al. found that compared to the placebo group, study participants randomized to the 7 and 20 µg/day calcifediol but not the vitamin D<sub>3</sub> groups had significantly lower serum PTH levels after



5 and 10 weeks of supplementation [53]. Similarly, Navarro-Valverde et al. demonstrated that, after 12 months of supplementation, study participants receiving calcifediol (20 µg/day, 266 µg/week, or 266 µg/2weeks) had lower PTH levels than those receiving vitamin D<sub>3</sub> (20 µg/day) [55]. Several studies revealed trends toward greater PTH reduction with calcifediol versus vitamin D<sub>3</sub>, but that difference did not reach statistical significance. For example, Bischoff-Ferrari et al. showed that over a 4-month period, PTH decreased by 18.1 pg/mL in study participants taking 20 µg/day of calcifediol versus 5.7 pg/mL in those taking 20 µg/day of vitamin D<sub>3</sub> [54]. Similarly, Vaes et al. reported that after 24 weeks of supplementation, there was a trend toward lower mean PTH levels in the calcifediol 15 µg/day treatment group (36.7 pg/mL) versus the vitamin D<sub>3</sub> 20 µg/day group (44.3 pg/mL) [56].

Plausibly, calcifediol would be superior to parent vitamin D<sub>3</sub> for suppressing PTH production. Beyond its ability to indirectly downregulate PTH secretion via the VDR and CASR (by promoting more renal 1,25(OH)<sub>2</sub>D<sub>3</sub> production and intestinal calcium absorption), calcifediol may have the capacity to directly suppress PTH production via entry into the parathyroid cell followed by its subsequent local conversion to 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>-VDR-mediated suppression of *PTH* expression [61–65]. Notably, none of the above studies were specifically designed or powered to test whether calcifediol is better than parent vitamin D at reducing circulating PTH levels. RCTs should specifically examine the comparative effects of calcifediol versus parent vitamin D on PTH levels in patients with 25(OH)D insufficiency and SHPT (see section “Calcifediol treatment of secondary hyperparathyroidism associated with chronic kidney disease” below).

### 3. Calcifediol treatment of secondary hyperparathyroidism associated with chronic kidney disease

#### 3.1 Pathophysiology of secondary hyperparathyroidism (SHPT)

SHPT often develops with advancing CKD in association with vitamin D insufficiency (VDI), usually defined as a serum total 25(OH)D of <30 ng/mL or 75 nmol/L. CKD afflicts 14.4% of the United States (US) adult population [66] and approximately 9.1% of the global population [67] and is driven by aging, obesity and the associated complications of hypertension and adult-onset diabetes [68]. SHPT is characterized by hypertrophy of the parathyroid glands, excessive production of PTH, and prolonged release of calcium and phosphorus from bone, leading to CKD-mineral

and bone disorder (MBD) including vascular calcification, the main cause of morbidity and mortality in CKD [69]. The majority of patients with stage 3–4 CKD develop VDI. When CKD and VDI are present in the same patient, the metabolism of the vitamin D pro-hormone (25(OH)D) to active hormone (1,25(OH)<sub>2</sub>D) by CYP27B1 in the kidney declines, causing low levels of serum 1,25(OH)<sub>2</sub>D. Declining circulating 1,25(OH)<sub>2</sub>D concentrations leading to hypocalcemia are associated with an increase in PTH secretion by the parathyroid glands [70]. In other tissues containing CYP27B1 (e.g., in disease-activated macrophages; see Table 73.1), diminished levels of 25(OH)D limit the local production of intracrine 1,25(OH)<sub>2</sub>D.

#### 3.2 Treatment of vitamin D insufficiency (VDI) in chronic kidney disease (CKD)

Supplementation with either cholecalciferol (vitamin D<sub>3</sub>) or ergocalciferol (vitamin D<sub>2</sub>) is recommended by both the Kidney/Disease Outcomes Quality Initiative (K/DOQI) and the Kidney Disease Improving Global Outcomes (KDIGO) Clinical Practice Guidelines [71–73] to address SHPT arising from VDI in patients with CKD. Supplementation is widely used despite a lack of expert consensus regarding the optimal dosing regimen or its effectiveness for reaching an appropriate target level of serum 25(OH)D to lower elevated PTH levels [39,73–76]. In the US and certain European countries, extended-release calcifediol (ERC) is approved to treat SHPT at a dose of 30 µg/day escalating, as needed, to 60 µg/day. In some European countries, immediate-release calcifediol (IRC) is approved to treat VDI associated with renal osteodystrophy, rickets in children, osteomalacia in adults and hypocalcemia.

Raising the serum 25(OH)D sufficiently in order to lower PTH in overweight patients with normal renal function is difficult with ergocalciferol or cholecalciferol [77], supporting the Endocrine Society’s recommended administration of two to three times more of these supplements than for patients with normal body weight [36]. VDI is highly prevalent in obese patients [78–80] consistent with the known inverse relationship between serum 25(OH)D levels and increased body weight or body mass index (BMI) [81,82]. Some studies have reported that CKD patients with severe VDI have the highest BMI values [83] and are more often morbidly obese [84].

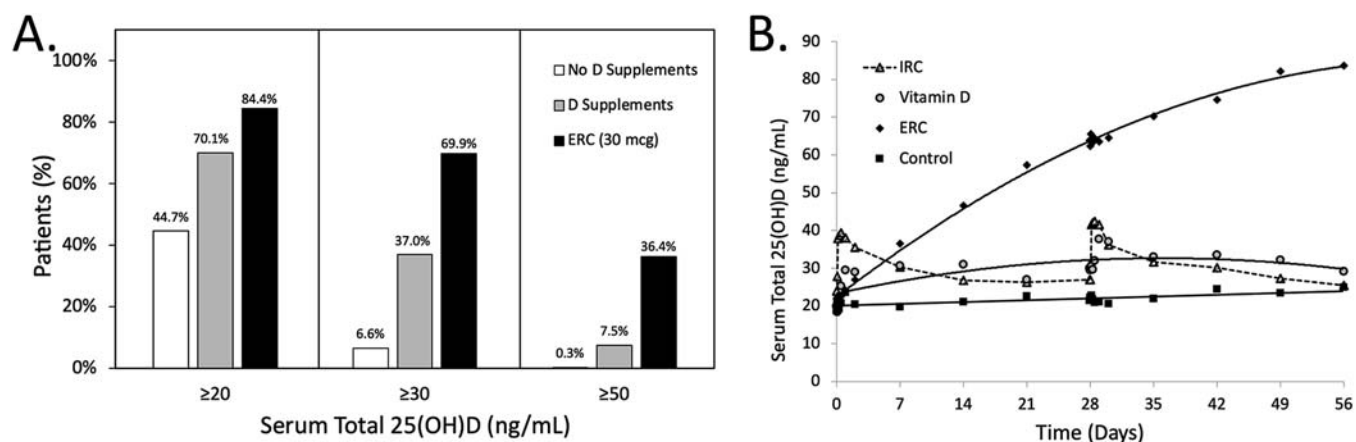
Recent evidence [39,85] has confirmed the limited effectiveness of vitamin D supplements in CKD patients, most of whom are overweight, supporting the alternative use of ERC. Data extracted from Fresenius Medical Care’s European database (EuCliD5) for 2459 adult non-dialysis CKD patients showed that only 41% had serum

25(OH)D levels of  $\geq 30$  ng/mL despite receiving standard of care treatment with parent vitamin D supplements. Only 7% had levels  $\geq 50$  ng/mL, a threshold newly identified in RCTs as required for clinically meaningful reductions in elevated PTH and serum bone turnover markers in CKD [22]. The observed negative inverse correlations of serum 25(OH)D levels with both BMI and body weight suggest that obesity can prevent routine vitamin D supplementation from raising serum 25(OH)D to targeted levels in CKD. Additional real-world data (Fig. 73.1, Panel A) have been collected in the US from a cohort of 321 adults with stage 3–4 CKD and VDI treated with cholecalciferol ( $n = 50$ ), ergocalciferol ( $n = 97$ ) or ERC ( $n = 174$ ) [85A]. Mean ( $\pm$ SE) serum 25(OH)D rose from  $19.6 \pm 8.2$  ng/mL to levels of  $\geq 30$  or  $\geq 50$  ng/mL in only 37.0% or 7.5% of patients, respectively, treated with cholecalciferol or ergocalciferol at monthly doses of  $\geq 50,000$  international units (IU) (64.7%), 14,000 to  $<50,000$  IU (23.1%) or 9000 to  $<14,000$  IU (12.2%). In contrast, 69.9% and 36.4% of patients attained these same 25(OH)D thresholds, respectively, treated with ERC at either 30 ( $n = 171$ ) or 60 ( $n = 3$ )  $\mu$ g/day.

A newly completed prospective RCT [86] evaluated the effectiveness of 8 weeks of oral treatment with cholecalciferol (300,000 IU/month;  $n = 16$ ), IRC (266  $\mu$ g/month;  $n = 15$ ) and ERC (60  $\mu$ g/day;  $n = 17$ ) versus a control group in raising serum 25(OH)D to targets of  $\geq 30$  or  $\geq 50$  ng/mL in patients with SHPT, VDI, and stages 3–4 CKD (Fig. 73.1, Panel B). Body weight was balanced among the treatment groups and mean ( $\pm$ SD) BMI was  $35.1 \pm 7.8$  kg/m<sup>2</sup>. The selected dose of oral cholecalciferol was the highest one routinely used

in adults with CKD; few clinicians would have recommended using more. The selected dose of IRC was an approved oral unit dose routinely used in Spain for CKD patients. The dose of 60  $\mu$ g/day of oral ERC was the highest approved dose and was selected to accelerate the timing under which the serum 25(OH)D targets could be attained, as normal dose titration (from 30 to 60  $\mu$ g/day) would have required a 6-month treatment period since steady-state serum levels of 25(OH)D would not have been achieved until after 3 months for any given dose. Mean ( $\pm$ SD) serum 25(OH)D gradually surpassed 50 ng/mL in 100% of subjects in the ERC group, reaching  $61.2 \pm 13.6$  ng/mL after 4 weeks of treatment and  $82.9 \pm 16.8$  ng/mL after 8 weeks. In the cholecalciferol group, mean 25(OH)D levels rose to only  $29.1 \pm 11.4$  ng/mL after 4 weeks and  $30.3 \pm 11.8$  ng/mL after 8 weeks, leaving 56% of subjects below the 30 ng/mL threshold. In the IRC group, mean 25(OH)D transiently reached the range of 37–43 ng/mL but declined below 30 ng/mL between doses. Serum 25(OH)D was unchanged in the control group. No subjects achieved serum 25(OH)D levels at or above the 50 ng/mL threshold with cholecalciferol or IRC treatment. Serum 25(OH)D levels in all groups at the end of treatment showed significant inverse relationships with body weight. ERC treatment produced 10%, 20%, and 30% reductions in plasma PTH from pretreatment baseline in 76.5%, 70.6%, and 41.2% of subjects, respectively, compared with 56.3%, 37.5%, and 25.0% for cholecalciferol treatment and 33.1%, 20.0%, and 6.7% for IRC treatment.

Data from this RCT support and extend those reported previously from an open-label RCT conducted



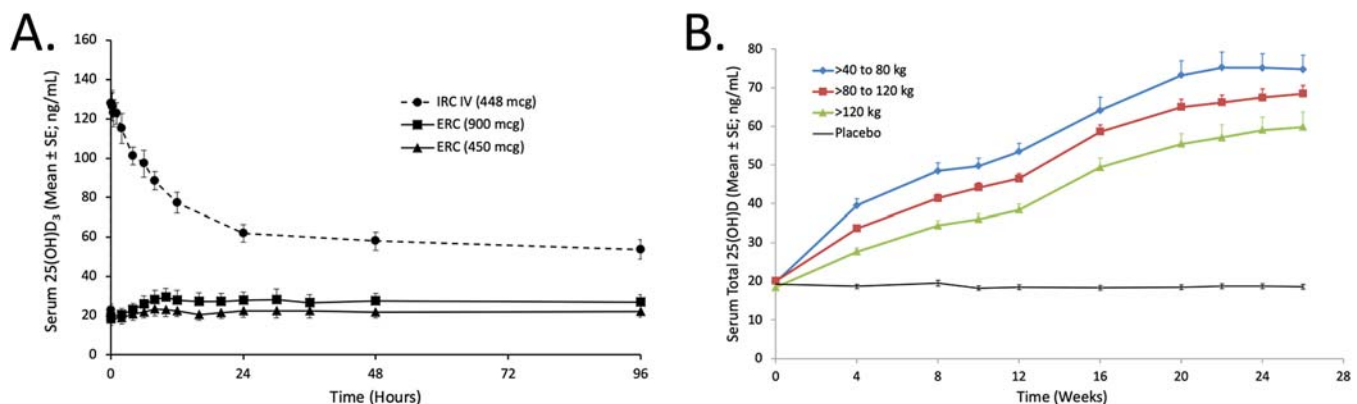
**FIGURE 73.1** Panel A shows the percentages of 321 adult chronic kidney disease (CKD) patients who achieved serum total 25-hydroxyvitamin D (25(OH)D) levels of  $\geq 20$ ,  $\geq 30$ , or  $\geq 50$  ng/mL with standard of care vitamin D repletion therapy in a real-world study in the US. These patients were treated with oral cholecalciferol (vitamin D<sub>3</sub>;  $n = 50$ ), ergocalciferol (vitamin D<sub>2</sub>;  $n = 97$ ) or extended-release calcifediol (ERC;  $n = 174$ ). Panel B depicts the mean serum total 25(OH)D levels achieved in a four-arm US RCT conducted in 69 patients with secondary hyperparathyroidism, stage 3 or 4 CKD and vitamin D insufficiency (mean 25(OH)D  $\pm$ SD  $20.6 \pm 6.6$  ng/mL) evaluating oral immediate-release calcifediol (IRC; 266  $\mu$ g/month), oral cholecalciferol (vitamin D<sub>3</sub>; 300,000 IU/month), oral ERC (60  $\mu$ g daily) versus a control group. Adapted and reprinted with permission from Ref. [86].

in a similar patient population which compared single oral ERC doses of 450 or 900  $\mu\text{g}$  to a single intravenous (IV) IRC dose of 448  $\mu\text{g}$  [87]. In that study, the 900  $\mu\text{g}$  ERC dose produced greater and more sustained PTH suppression compared to IRC despite lower bioavailability (25% vs. 100%) and 25(OH)D exposure. Mean percent reductions in PTH from baseline were minimal over the postdose period for both IRC and the lower ERC dose but reached approximately 20% for the higher ERC dose ( $P < .05$ ). As depicted in **Panel A** of [Fig. 73.2](#), the maximum concentration ( $C_{\text{max}}$ ) of serum 25(OH)D was 134 ng/mL at 0.5 h after IV administration of IRC, 33 ng/mL after 13.6 h for the higher oral ERC dose, and 25 ng/mL after 13.1 h for the lower oral ERC dose and. Subsequent studies with ERC in predialysis patients have demonstrated that PTH-lowering responses are directly proportional to the degree to which serum 25(OH)D is gradually (not suddenly) elevated, with clinically meaningful responses occurring when levels surpassed 50 ng/mL [22].

Other studies in predialysis patients have shown that oral IRC failed to produce clinically meaningful reductions in PTH ( $>30\%$  from pretreatment baseline) unless administered at doses that raised mean serum 25(OH)D to levels approaching or exceeding 100 ng/mL, the upper limit of the laboratory normal range. In one study [88], subjects were treated with oral IRC (10–50  $\mu\text{g}/\text{day}$ ) or calcium carbonate (control) for 2 years. PTH responses in the two treatment groups were reported as “comparable” and, in aggregate, PTH decreased by only 4%. Another study [89] reported that a much higher oral dose of IRC (160  $\mu\text{g}/\text{day}$ ) reduced PTH by only 6%. A third study [90] reported a mean decrease in PTH of 21%, but only at oral IRC doses of 200  $\mu\text{g}/\text{day}$  or IV doses of 500  $\mu\text{g}$  every 1–5 days. Although serum

25(OH)D levels in this third study were not reported, such high doses most certainly produced sustained levels far above 100 ng/mL. In a further study [91], 125  $\mu\text{g}$  of oral IRC administered to 29 subjects thrice weekly for 6 months yielded a mean PTH decline from 99.5 to 60.3 pg/mL ( $-39\%$ ) with serum 25(OH)D exceeding 100 ng/mL in nearly half of the subjects. Five subjects treated with IRC experienced adverse cardiovascular events, one developed hypercalcemia and one died versus none in a parallel placebo control group. Taken together, data from these previous studies and from the current study support the conclusion that gradual delivery of calcifediol in the form of ERC allows effective treatment of SHPT in CKD patients without abruptly and excessively raising serum 25(OH)D to calcemic levels.

Longer time course data for serum total 25(OH)D and plasma PTH with daily ERC treatment or placebo were examined in 429 adult patients with SHPT, stages 3–4 CKD, and VDI as a function of body weight in pooled data from two identical, concurrent prospective US RCTs [21]. Of these subjects, 356 completed the 20- to 26-week treatment period per-protocol and were included in the analysis. Enrolled subjects assigned to ERC treatment ( $n = 234$ ) ingested one 30  $\mu\text{g}$  capsule daily for 12 weeks followed by one or two capsules (30 or 60  $\mu\text{g}$ ) daily for an additional 14 weeks. Control subjects ( $n = 122$ ) received matching placebo capsules. Mean ( $\pm\text{SD}$ ) serum 25(OH)D remained unchanged with placebo treatment but rose progressively with ERC treatment to  $67.1 \pm 21.6$  ( $\pm\text{SD}$ ) ng/mL (mean of weeks 20–26). Mean serum 25(OH)D at the end of treatment was inversely related to body weight but exceeded 50 ng/mL in all weight categories ([Fig. 73.2, Panel B](#)). Side effects observed at these levels of exposure were



**FIGURE 73.2** **Panel A** compares the effects of bolus intravenous (IV) immediate-release calcifediol (IRC; 448  $\mu\text{g}$ , circles,  $n = 10$ ) and two different doses of oral extended-release calcifediol (ERC; 450  $\mu\text{g}$ , triangles,  $n = 9$  and 900  $\mu\text{g}$ , squares,  $n = 9$ ) on the mean  $\pm$  SE serum 25(OH)D in 28 adults with stage 3–4 chronic kidney disease, secondary hyperparathyroidism (SHPT) and vitamin D insufficiency (VDI; 25(OH)D  $< 30$  ng/mL). **Panel B** shows the time course of mean  $\pm$  SE serum 25OHD levels by body weight category in 356 adults with SHPT, stage 3–4 and VDI (25(OH)D  $< 30$  ng/mL) who were treated with ERC (30  $\mu\text{g}$  escalating as needed to 60  $\mu\text{g}/\text{day}$ ;  $n = 234$ ) or placebo ( $n = 122$ ) for 6 months. Reprinted with permission from Ref. [87].

similar to placebo. ERC reduced plasma PTH by at least 10% in 72% of subjects and by at least 30% in 40% of subjects, rising to 50% of subjects with longer treatment, irrespective of CKD stage or degree of obesity.

In CKD patients, oral calcitriol or another  $1\alpha$ -hydroxylated vitamin D analog is often used in place of vitamin D supplementation when PTH levels rise and remain persistently above the normal range with advancing disease. The original KDIGO clinical practice guideline for the treatment of CKD-MBD recommended this approach in patients with stage 3–4 CKD [72], consistent with the widely held view that  $1\alpha$ -hydroxylated drugs become necessary in later stages of CKD due to the loss of renal CYP27B1 activity. The more recent KDIGO guideline update [73] reversed this recommendation, suggesting instead that  $1\alpha$ -hydroxylated vitamin D hormone therapy should no longer be routinely used in nondialysis CKD due to the increased risk of hypercalcemia. The updated KDIGO guideline also highlighted the uncertain effectiveness of supplements for suppressing elevated PTH, leaving nephrologists without useful guidance on how best to treat SHPT in CKD patients. Several meta-analyses of RCTs conducted in CKD patients have shown that ergocalciferol and cholecalciferol supplementation are unreliable in achieving a serum 25(OH)D target of  $\geq 30$  ng/mL, let alone  $\geq 50$  ng/mL [39,74,75,92]. One meta-analysis [39] concluded that parent vitamin D supplementation produced such large variations in response that a precise evaluation of the true treatment effect was difficult, especially in overweight patients. When PTH rises in advancing CKD, patients are usually switched to calcitriol or another  $1\alpha$ -hydroxylated vitamin D analog with the justification that too much renal CYP27B1 has been lost, preventing the sufficient conversion of 25(OH)D to hormone. Switching, however, is inappropriate because PTH control in nondialysis patients can be accomplished by raising serum 25(OH)D safely to  $\geq 50$  ng/mL with ERC, irrespective of CKD stage and obesity, allowing for sufficient intracrine calcitriol generation extra-renal to effectively treat SHPT.

As noted previously, most CKD patients [68,93] are overweight or obese, a problem that drives disease progression through the comorbidities of type 2 diabetes and hypertension. Cholecalciferol and ergocalciferol are both fat-soluble molecules which accumulate preferentially in adipose tissue [94,95]. Both have low affinities for DBP, are poorly drawn out of adipose into circulation for hepatic activation [49,94], and are prone to in situ catabolism by the CYP24A1, the vitamin D catabolic enzyme encoded by the CYP24A1 gene that can be upregulated in earlier stages of CKD. Hepatic 25-hydroxylase activity, primarily through CYP2R1, is reduced in both obesity [47] and CKD [49,50], blunting the intended elevation of serum 25(OH)D. In contrast,

25(OH)D requires no hepatic activation, is more water soluble, and avidly binds DBP, reducing accumulation in adipose tissue and enabling its circulation to extrarenal tissues containing CYP27B1 and conversion to intracrine calcitriol [96]. Gradual delivery of 25(OH)D in the form of ERC has been shown to cause minimal suppression of extra-renal CYP27B1 and minimal upregulation of CYP24A1 [97].

Production of intracrine calcitriol, as occurs in the parathyroids and activated innate immune cells, depends on an adequate supply of 25(OH)D, fostering an ongoing dialogue about the optimum level for serum 25(OH)D in patients with stage 3–4 CKD. RCTs conducted with ERC suggest that this target should be at least 50 ng/mL [22], not 20 or 30 ng/mL endorsed by clinical practice guidelines for bone health [35,36,71], to enable the production of intracrine calcitriol and control of SHPT despite the progressive loss of renal CYP27B1 activity in advancing CKD. In fact, production of intracrine calcitriol at serum 25(OH)D levels  $\geq 50$  ng/mL has been documented in anephric patients [96] and in hemodialysis patients with intact residual kidneys [98]. As will be discussed in the next section, “Calcifediol treatment of microbial disease to boost the human innate and adaptive immune responses” and as shown in Table 73.1, the most prolific source of extra-renal  $1,25(\text{OH})_2\text{D}$  is the human monocyte-macrophage.

#### 4. Calcifediol treatment of microbial disease to boost the human innate and adaptive immune responses

##### 4.1 The concerted, serial link among calcifediol, CYP27B1, calcitriol, the vitamin D receptor (VDR), and the human immune response

This link was initially supported by a set of findings made in the early 1980s, before the actual cloning of either the human VDR or CYP27B1. First was the observation that a patient without kidneys suffering from sarcoidosis, an idiopathic, human, tissue macrophage proliferative disease primarily of the lung, could develop  $1,25(\text{OH})_2\text{D}$ -mediated hypercalcemia without any renal vitamin D-1-hydroxylating activity [99]. Second was the discovery that alveolar macrophages harvested from the lungs of patients with active sarcoidosis were capable of prolific  $1,25(\text{OH})_2\text{D}_3$  production ex vivo when exposed to physiologically relevant concentrations of 25(OH)D<sub>3</sub> in the culture medium [3]. Third were the findings that, when activated ex vivo by mitogenic lectins or Epstein–Barr virus, human peripheral mononuclear cells, including monocytes, T-lymphocytes, and B-lymphocytes,



expressed a high-affinity receptor-like molecule for  $1,25(\text{OH})_2\text{D}$  [100], later proven to be the VDR, and that when bound to  $1,25(\text{OH})_2\text{D}$  resulted in inhibition of the proliferation and biological activity of that immune cell [101].

Subsequently, other key discoveries were made that supported the immunoactions of vitamin D. These included the finding that metabolism of  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$  was the result of induced expression of the CYP27B1 by cells of the human innate immune response, including monocytes, macrophages and dendritic cells [102]. This event was supported by the finding that extra-renal overproduction of  $1,25(\text{OH})_2\text{D}$  of sufficient quantity to cause hypercalcemia in the host was observed in over 20 different granuloma-forming diseases of infectious (e.g., tuberculosis), noninfectious (e.g., silicone induced) and neoplastic (AIDS-associated B-cell lymphoma) origin [103]. In all of these diseases, the synthetic source of  $1,25(\text{OH})_2\text{D}$  was considered to be the tissue macrophage, extra-renal in nature and not bound by factors/events that normally controlled the renal  $1,25(\text{OH})_2\text{D}$  production (see Table 73.1). Hypercalcemia in patients with active granuloma-forming disease could be induced when they were challenged with increased sunlight exposure or dosed with vitamin D, leading to a subsequent increase in serum  $25(\text{OH})\text{D}$  levels [13,14]. Next was the discovery that the downstream immune action of  $1,25(\text{OH})_2\text{D}$  was mediated through upregulated expression of the VDR in antigen-activated innate and adaptive immune cells in local inflammatory microenvironments, especially in the lung [104]. Last was recognition of the fact that the link between the local production and immune action of calcitriol is largely mediated through the unique ability of human and higher primate monocytes/macrophages and dendritic cells to transactivate antimicrobial genes (e.g., *cathelicidin antimicrobial peptide* [CAMP]) through the  $1,25(\text{OH})_2\text{D}$ -activated VDR ([105]; see Chapters 9 and 94).

After cleavage from its 18 KD precursor form (hCAP18) inside the innate immune cell, the cationic cleavage peptide, LL-37, becomes the bioactive product of the CAMP gene. LL-37 forms an amphipathic  $\alpha$ -helix when it comes in contact with hydrophobic surfaces, such as microbial membranes, disrupting the ordered arrangement and function of the hydrophobic phospholipid components of the membrane [106,107]. Increases in circulating levels of LL-37 have been observed in bacterial [108], mycobacterial [109–112], and viral [113] infections. Importantly, local concentrations of LL-37 have been observed at the site of infection [114,115].  $1,25(\text{OH})_2\text{D}$  regulation of the production and action of LL-37 to kill microbes, either intracellularly or extracellularly after secretion from an innate immune cell, turns out to be a completely fortuitous event in

nature. It is the consequence of the random insertion of a transposable Alu repeat element containing a positive, cis-acting, consensus vitamin D responsive element (VDRE) in the human CAMP promoter [105]. The end result is  $25(\text{OH})\text{D}$ -driven, intracrine production of  $1,25(\text{OH})_2\text{D}$  via upregulated expression of the CYP27B1 in TLR-activated, VDR-expressing innate immune cells with subsequent transactivation of the CAMP gene.

If the above slate of concerted immune response events is true, then the following set of observations should hold clinically. First, local concentrations of  $1,25(\text{OH})_2\text{D}$  at the site of microbial infection and TLR activation of disease-activated macrophages in patients with macrophage proliferative disease should be increased compared to the concentration found in the general circulation. Proof of this reality was the finding of a steep increase in the pleural fluid:serum concentration of free  $1,25(\text{OH})_2\text{D}$  in patients with active pulmonary tuberculosis [103,104]. Second, the amount of locally produced  $1,25(\text{OH})_2\text{D}$  should be dependent on the concentration of substrate  $25(\text{OH})\text{D}$  available to the intracrine-acting CYP27B1. Proof of this dose-response reality was shown to be the case for alveolar macrophages harvested from patients with the active granuloma-forming disease [3,27]. Third, the production of LL-37 should be positively correlated with  $25(\text{OH})\text{D}$ -driven,  $1,25(\text{OH})_2\text{D}$ -VDR-mediated intracrine expression of the CAMP gene and its bioactive antimicrobial product LL-37. Initial proof of this was demonstrated when serum from vitamin D insufficient subjects, before and after restoration of the serum  $25(\text{OH})\text{D}$  level to normal, was used to condition activated, heterologous [29], or homologous [116] primary human monocyte-macrophages to increase LL-37 production. And fourth, an increase in the amount of circulating  $25(\text{OH})\text{D}$  substrate available to the activated CYP27B1 in human disease-activated macrophages should result in improved clinical outcomes in infected patients. There is now mounting evidence, including from large-scale meta-analyses, that is the case, especially in patients with acute respiratory infections (ARI) of viral origin [117–121] including those with COVID-19 [122].

## 4.2 Microbial infection and the serum $25(\text{OH})\text{D}$ status of the host

Again, it stands to reason that if the above set of circumstances is true, then insufficient  $25(\text{OH})\text{D}$  status of the human host, manifest by low circulating levels of the metabolite (usually defined as a total serum  $25(\text{OH})\text{D}$  level  $<20$  ng/mL or  $70$  nmol/L) should be associated with increased susceptibility to microbial

disease. Indeed, insufficient 25(OH)D in serum has been associated with increased rates of influenza [123], viral upper respiratory tract infection [124,125], pneumonia [126], HIV [127,128], and active tuberculosis [129]. The earliest attempt to reduce the incidence and/or severity of microbial infection with vitamin D dates back to the early twentieth century, when sunlight exposure was used to treat patients with tuberculosis [130]. Despite these early findings, the administration of vitamin D and its metabolites in RCTs of patients with infectious and/or inflammatory diseases was not pursued until almost a century later. The results of these more recent trials have been mixed. The strongest evidence of a vitamin D antimicrobial effect arose from effective supplementation studies in the context of influenza [118] and upper respiratory tract infections [120,131]. Since the onset of the COVID-19 pandemic, VDI has been associated with increased SARS-CoV-2 infection risk [132,133], greater COVID-19 severity [134], and delayed COVID-19 recovery [135], prompting supplementation studies that, although variable in power and design, have shown a reduced requirement for intensive care unit (ICU) treatment among patients hospitalized with COVID-19 [136] and accelerated recovery from pulmonary symptoms in nonhospitalized SARS-CoV-2-infected patients [40].

#### 4.3 Use of immediate-release calcifediol (IRC) and extended-release calcifediol (ERC) in acute respiratory infections (ARI) including COVID-19

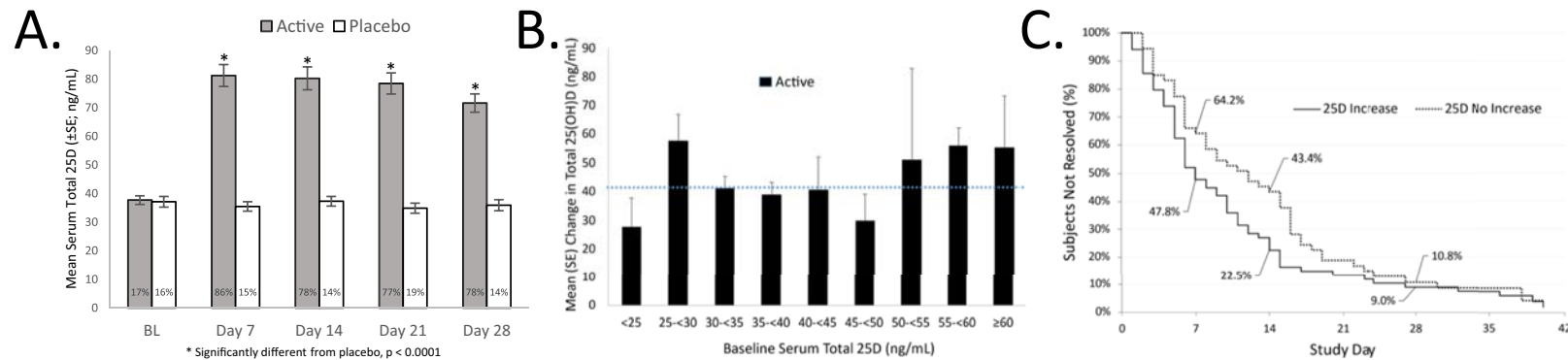
Results on the use of IRC in the prevention and treatment of COVID-19 are just starting to appear (e.g., [135,137–139]) with a few trials still ongoing. All indicate that treatment with calcifediol slows the onset and/or reduces adverse outcomes in COVID-19 disease. The problem with these studies is that they were not performed in a fully prospective, randomized, double-blind, placebo-controlled manner.

To fill this knowledge gap, we performed a pilot RCT to investigate the effect of raising serum total 25(OH)D levels to the range of 50–100 ng/mL with oral extended-release calcifediol (ERC) on time to symptom resolution in outpatients with mild to moderate COVID-19 [40]. The study focused on outpatients rather than on hospitalized patients because effective early intervention could prevent hospitalizations. The trial was a prospective, multicenter, randomized, double-blind, placebo-controlled study comparing ERC to placebo treatment (1:1) of 160 outpatients with COVID-19 enrolled between November 2020 and October 2021. Subjects were treated for 4 weeks with ERC 30 µg/capsules, 300 µg on Days 1–3 and 60 µg on Days 4–27) or

placebo. Primary endpoints were raising serum 25(OH)D to  $\geq 50$  ng/mL and resolution time for aggregated self-reported symptoms. Secondary endpoints included symptom resolution time versus the serum 25(OH)D level. A total of 171 subjects were randomized, 160 were treated, and 134 (65 ERC and 69 placebo) retained to the completion of the trial (per-protocol population). Average age (43 years), gender (59% female), racial makeup (80% Hispanic, 12% White, 7% African American, 1% Other), BMI (76% overweight and 40% obese), and comorbidities (26%) that increase susceptibility to clinical COVID-19 were not significantly different between the ERC and placebo groups.

The mean ( $\pm$ SE) baseline serum 25(OH)D was in the “normal” range for bone health ( $\geq 30$  ng/mL) in both the ERC-treated and placebo groups ( $37 \pm 1$  ng/mL) (Fig. 73.3, Panel A). This regimen of ERC increased the mean total serum 25(OH)D to  $82 \pm 4$  ng/mL by Day 7 of treatment ( $P < .0001$ ) and caused 86% of ERC-treated subjects to attain a 25(OH)D level  $\geq 50$  ng/mL. The corresponding level in the placebo-treated group was significantly lower at all evaluation time points and unchanged over the course of the trial (Fig. 73.3, Panel A). Regardless of the starting serum 25(OH)D level, ERC-treated subjects had significant increases in their 25(OH)D ( $P < .005$ ) by the end of the 4-week treatment period with no significant correlation between the magnitude of the ERC-induced increase in serum 25(OH)D based on the subject’s starting 25(OH)D level (Fig. 73.3, Panel B). Resolution time for five aggregated COVID-19 symptoms was unchanged by ERC, because two composite nonrespiratory symptoms (body aches/pains, chills/shivering) were unchanged by either ERC or placebo treatment. Prespecified analyses showed that the triad pulmonary symptoms, trouble breathing, chest congestion, and dry or hacking cough, tended to resolve earlier when serum 25(OH)D levels reached  $\geq 50$  ng/mL, but statistical significance was limited by small sample size and noncompliance; serum 25(OH)D increased in seven placebo subjects (unauthorized supplementation) but not in five ERC subjects (failure to dose).

A post hoc analysis of results from subjects in the per-protocol population demonstrated that respiratory symptoms resolved 3.0 days faster when 25(OH)D was elevated at Days 7 and 14 ( $P < .05$ ; Fig. 73.3, Panel A). Chest congestion, in particular, resolved 4.0 days faster with net 25(OH)D increases of  $\geq 25$  ng/mL ( $P < .05$ ). All of these significant changes in symptom resolution occurred in the first 2 weeks of ERC treatment. Serum 25(OH)D levels of  $\geq 50$  ng/mL have been suggested to support intracellular generation of 1,25(OH)<sub>2</sub>D, activation of the VDR, transactivation of the CAMP gene, and production and release of LL37 to combat SARS-CoV-2 proliferation in the host [107]. Compared to no



**FIGURE 73.3** **Panel A** shows the mean ( $\pm$ SE) total serum 25-hydroxyvitamin D (25(OH)D) by study day and per-protocol treatment group (closed bars: extended-release calcifediol [ERC]; open bars: placebo). Percentages at the base of each bar indicate the proportion of subjects achieving serum 25(OH)D levels of at least 50 ng/mL. Asterisks indicate significant differences between treatment groups ( $P < .0001$ ). **Panel B** depicts the mean ( $\pm$ SE) increases in serum total 25(OH)D during ERC treatment by baseline serum 25(OH)D in the per-protocol population. The horizontal dotted line indicates the mean increase for all treated subjects. All increases were significant ( $P < .005$ ). **Panel C** displays Kaplan–Meier curves of the time to resolution of a composite of three pulmonary symptoms (trouble breathing, chest congestion, dry or hacking cough) in subjects in the per-protocol population achieving increases versus no increases in the serum total 25(OH)D level. The difference between the plotted curves is significant ( $P < .05$ ). Reprinted with permission from Ref. [40].

change in placebo-treated subjects, ERC-treated subjects showed a significant increase in serum calcitriol ( $P < .005$ ) and a 10% increase in the serum LL37 level at the conclusion of the 4-week trial. It is possible that these elevations in the circulation reflect their elevated concentrations in the extravascular inflammatory micro-environment of the lung.

This study's inability to show more significant differences in time to resolution for respiratory symptoms is likely due to (i) the small sample size of the pilot, (ii) the fact that 17% of subjects assigned to placebo treatment unexpectedly had serum 25(OH)D levels  $\geq 50$  ng/mL at baseline, (iii) dosing noncompliance, and (iv) delays between onset of symptoms and diagnosis of COVID-19 and between diagnosis and initiation of treatment. The observed positive effect of elevation of the serum 25(OH)D on the resolution of respiratory symptoms is based on a post hoc analysis and needs confirmation in a larger study. Despite these issues, the study recorded some interesting results. For example, aggressive and persistent elevation of the serum 25(OH)D into the 50–100 ng/mL range for 4 weeks with ERC was without calcemic side effects of any sort. This indicates that it is safe to gradually drive serum 25(OH)D levels with ERC to the range where the local extra-renal production of 1,25(OH)<sub>2</sub>D occurs (see Chapter 11) for weeks at a time when antimicrobial activity is most needed for ARI, including COVID-19. Also interesting is the fact that pulmonary symptom resolution was not predicated on the subject being vitamin D insufficient at the beginning of the study, suggesting that it was secondary to the robust incremental increase in the serum 25(OH)D level achieved with ERC treatment.

This study with ERC differed from previous evaluations of vitamin D supplementation in COVID-19, primarily because it was designed to target a high serum 25(OH)D exposure (50–100 ng/mL) with downward dose adjustment, as needed. The known adverse impact of adipose tissue on 25(OH)D bioavailability with vitamin D supplements is readily overcome with ERC [85]. Prescription and nonprescription supplements of cholecalciferol and ergocalciferol are fat-soluble, poorly absorbed in the intestine, accumulate preferentially in adipose tissue [140], possess low affinities for serum DBP [141] and are poorly mobilized from adipose into circulation for hepatic activation [47,48]. Such supplements have multi-day delays in raising the serum 25(OH)D level and prove to be unreliable in raising 25(OH)D in overweight or obese patients who are at high risk for COVID-19 [77,95]. By contrast, calcifediol requires no hepatic activation, is more water soluble, and avidly binds to DBP, reducing accumulation in adipose tissue and enabling ready availability to peripheral tissues, including virus-activated immune cells containing CYP27B1. Collectively, this also means that

calcifediol dosing can be stopped abruptly with an expected decrease in the serum calcifediol level soon to follow; this sort of dose adjustment:rapid response relationship is not possible with treatment with cholecalciferol and ergocalciferol, especially when the adipose reservoir of the host is great.

Consistent with the excellent safety profile established for ERC in CKD patients [21], in this study, the slow-release formulation of ERC safely achieved high serum 25(OH)D exposure (50–100 ng/mL). Persistently elevated serum levels of 25(OH)D in this range have been of concern for vitamin D supplement administration [35]. Compounding this concern is the reported inverse relationship between the baseline 25(OH)D levels and observed increases seen with vitamin D supplementation regimens [142]. However, as previously stated, this relationship was not observed in this ERC trial, perhaps owing to the fact that the baseline serum 25(OH)D in both the ERC and placebo control groups were  $>30$  ng/mL. Safety endpoints and serum biomarkers of the calcemic actions of ERC treatment showed no clinically meaningful changes. Serum calcium and phosphorus, circulating PTH, and estimated glomerular filtration rate (eGFR) all remained stable. Episodes of hypercalcemia or hyperphosphatemia were not observed, even in subjects whose serum 25(OH)D exceeded 100 ng/mL during the course of the trial. This is perhaps not surprising as this was a short-term study aimed at altering the course of ARI with SARS-CoV2 infection.

The current ERC trial also varies from trials using IRC to boost the serum 25(OH)D level in COVID-19 subjects. The primary difference resides in the design of the pilot ERC study. Unlike all other reports [135,137–139], it was a prospective, randomized, double-blind, placebo-controlled trial that used an extended-release, not an immediate-release, formulation of calcifediol, avoiding the uncertain dose-response effect with IRC. Oral IRC results in a more rapid release of calcifediol in the proximal bowel for absorption and a lower achieved 25(OH)D level in the serum [139]. On the other hand, the release of calcifediol from ERC is gradual, continues over a period of 12 h (based on in vitro dissolution), and likely occurs primarily in the colon [21]. The conclusion from this randomized, double-blind, placebo-controlled pilot study was that daily oral administration of ERC (60  $\mu$ g), after a 3-day oral loading dose of 300  $\mu$ g, for 4 weeks was effective in rapidly, robustly, and safely increasing serum 25(OH)D in COVID-19 outpatients to levels supporting the immune cell synthesis and secretion of 1,25(OH)<sub>2</sub>D into the inflammatory microenvironment of the SARS-CoV2 infected lung (see Table 73.1) with the possibility to accelerate the resolution of respiratory symptoms and mitigation of pneumonia risk.



## 5. Conclusion

This chapter attempts to outline three dominant clinical situations in which the use of calcifediol may be superior to supplementation with ergocalciferol or cholecalciferol. These are: (1) endocrine-mediated SHPT associated with VDI (serum 25(OH)D < 20 ng/mL or 30 ng/mL) and bone loss; (2) more severe SHPT associated with CKD, relative VDI, and endocrine-mediated bone loss; and (3) microbial infections, particularly viral disease in the lung (e.g., influenza and COVID-19) where relatively high levels of 25(OH)D (50–100 ng/mL range) are required to satisfy the substrate needs of the CYP27B1 expressed in activated innate immune cells (e.g., macrophages) in order to drive 1,25(OH)<sub>2</sub>D-VDR-anti-microbial response in the local, tissue-based inflammatory microenvironment.

## 6. Summary points

- The key advantages of raising serum 25(OH)D with oral calcifediol using either immediate-release (IRC) or extended-release (ERC) preparations vs. oral vitamin D supplements can be summarized as follows: calcifediol (i) is bound with high affinity by the serum DBP which reduces the total body volume of distribution of this metabolite to adipose tissue, (ii) bypasses the need for hepatic 25-hydroxylation, and (iii) can raise serum 25(OH)D more effectively.
- Among orally administered vitamin D repletion therapies, ERC appears to be safer and more effective than IRC in raising serum 25(OH)D to higher targets (e.g., ≥50 ng/mL), and both calcifediol formulations (ERC and IRC) are far more effective than vitamin D supplements (ergocalciferol or cholecalciferol). More comparative data from RCTs are needed for confirmation.
- Aggressive, short-term, oral supplementation with calcifediol can significantly increase serum 25(OH)D and potentially improve outcomes in subjects with acute, viral upper respiratory tract infection (e.g., influenza).
- In a pilot RCT, a blinded comparison of symptomatic, placebo-treated COVID-19 outpatients to those treated with ERC showed that ERC safely and effectively increased mean serum 25(OH)D levels to approximately 80 ng/mL and accelerated the mean resolution time for pulmonary symptoms by 3 days in the immediate 2-week treatment interval. This provided preliminary evidence for the safety and efficacy of ERC in the treatment of symptomatic COVID-19 especially early in the course of the disease.
- Increasing the serum 25(OH)D in symptomatic COVID-19 outpatients to 50–100 ng/mL, the “tissue immunoactive range,” with ERC appears to reduce pulmonary symptoms even in those patients who have a mean baseline 25(OH)D level in the normal range (≥30 ng/mL). This suggests that the immunoactions of calcifediol are related to the elevation of 25(OH)D to 50–100 ng/mL and are not confined to only those with VDI.

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## Further reading

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# Vitamin D and organ transplantation

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## OBJECTIVES

- Review the role of vitamin D in immune function, specifically as it relates to the transplant population.
- Describe the risk factors for vitamin D deficiency in patients with end stage pulmonary disease, liver failure, congestive heart failure, and chronic kidney disease prior to organ transplantation.
- Examine the prevalence of vitamin D deficiency at the time of, shortly after, and in long-term organ transplant recipients.
- Summarize interventional trials evaluating 1,25(OH)<sub>2</sub>D and its analogs for the prevention and treatment of bone loss following solid organ transplantation.

## 1. Introduction

There are many skeletal and extraskeletal sequelae of vitamin D deficiency among organ transplant patients. Vitamin D deficiency can result in secondary hyperparathyroidism, bone loss, and fracture, as well as muscle weakness and falls [1–3]. Insulin resistance (see [Chapters 75 and 76](#)), hypertension ([Chapter 41](#)), and malignancy ([Chapters 84–92](#)) are important extraskeletal sequelae [4–7]. The role of vitamin D in regulating immune function is of potential importance among transplant patients [8] (see [Chapters 94–106](#)). In this chapter, we will review the role of vitamin D in immune function as it relates to the transplant population. The prevalence of vitamin D deficiency in organ transplant

candidates and in long-term transplant recipients will be examined, with assessment of vitamin D status based on 25(OH)D measurements, currently the best available indicator of vitamin D stores. Finally, we will summarize interventional trials evaluating 1,25(OH)<sub>2</sub>D and its analogs for the prevention and treatment of bone loss following solid organ transplantation.

## 2. Effects of vitamin D on immunity and graft rejection

### 2.1 Role of vitamin D in allograft rejection

The role of vitamin D in the regulation of immune cell proliferation, differentiation, and responsiveness has been summarized in several reviews [9–14] and other chapters in this book ([Chapters 94–106](#)). In animal studies, administration of 1,25(OH)<sub>2</sub>D (calcitriol) prevented acute allograft rejection following kidney [15], heart [16], and liver [17,18] transplantation. In a recent animal study, administration of nebulized 1,25(OH)<sub>2</sub>D reduced inflammatory cells in the bronchoalveolar lavage [19]. Data from human studies have yielded mixed results. Pretransplant vitamin D insufficiency in pediatric liver transplant candidates was directly associated with T cell–mediated rejection during the first year posttransplant [20]. In retrospective studies of kidney transplant recipients, calcitriol supplementation was associated with fewer episodes of acute cellular rejection [21], reduced glucocorticoid requirements [22], and decreased expression of costimulatory and human leukocyte antigen–antigen D related (HLA-DR) molecules [23], suggesting a possible mechanism for improved allograft survival. Briffa et al. found that patients treated with 1,25(OH)<sub>2</sub>D (calcitriol) following

heart transplantation had a reduction in their requirement for cyclosporine, a calcineurin inhibitor that is a commonly used immunosuppressive medication [24]. However, in our clinical trial of heart transplant recipients treated with calcitriol, we did not observe lower cyclosporine or prednisone doses in patients randomized to calcitriol [25]. A recent prospective cohort study of adult kidney transplant recipients did not find an association between serum 25(OH)D or 1,25(OH)<sub>2</sub>D and risk of rejection following transplantation [26]. In a prospective-controlled study of liver transplant patients, calcitriol use was associated with both lower rates of acute rejection and an increase in regulatory T cells as assessed by flow cytometry, suggesting that calcitriol may mitigate acute rejection through enhancing T cell function [27]. Further prospective human studies are needed to explore the role of 1,25(OH)<sub>2</sub>D and parent vitamin D in prevention of graft rejection and infection after transplantation.

## 2.2 Immune effects of vitamin D

Interest in the role of vitamin D in immunity has burgeoned since the COVID-19 pandemic and has led to a surge in the number of publications on this topic, well beyond the scope of this chapter [28–31] (see Chapter 99). Monocytes and macrophages express the enzyme 1 $\alpha$ -hydroxylase (CYP27B1) and produce 1,25(OH)<sub>2</sub>D, which has intracellular antimicrobial effects and can also interact with and govern the cytokine profiles of activated T and B lymphocytes in the local environment (see Chapter 9) [32]. The ability of monocytes and macrophages to synthesize sufficient 1,25(OH)<sub>2</sub>D is dependent on availability of adequate serum concentrations of 25(OH)D and therefore increases in response to vitamin D supplementation [33]. When there is insufficient 25(OH)D available, immunity may be impaired because local production of 1,25(OH)<sub>2</sub>D will decline. Subsequent decreased binding of 1,25(OH)<sub>2</sub>D to the macrophage vitamin D receptor (VDR) will result in reduced antimicrobial activity against ingested microbes [32] (see Chapters 94 and 95). The antimicrobial actions of 1,25(OH)<sub>2</sub>D also occur in barrier epithelial cells of the skin [34,35], gut [36], colon [37], and lung [38–40], sites which may be of particular importance in transplant recipients. Vitamin D may also play an important role in mitigating oxidative stress in the liver and preserving hepatic function [41] (see Chapter 77). Vitamin D has been shown to protect against bacterial and viral infections as well as tuberculosis in many prior laboratory-based and observational clinical investigations [34], [42–49] (see Chapters 94 and 95). In nude mice, 1 $\alpha$ -hydroxyvitamin D (1 $\alpha$ -OHD, metabolized to 1,25(OH)<sub>2</sub>D by 25-hydroxylase)

inhibited rejection mediated by alloreactive CD4(+) memory T cells and prolonged cardiac allograft survival [50]. Research using genome-wide analyses suggests that both VDR and 1 $\alpha$ -hydroxylase expressions are increased in macrophages following a pathogen challenge [51,52]. Vitamin D may act through cytokine signaling via the extrarenal 1 $\alpha$ -hydroxylase to influence intracrine vitamin D pathways, which can enhance or abrogate the systemic response to 25(OH)D [51,53]. Vitamin D may also exert its effects at the epigenetic level by modulating microRNA function, which can affect the transcriptional regulation of gene expression [54,55] (see Chapters 12 and 14).

In a population-based study, upper respiratory tract infections were more common in individuals with lower 25(OH)D levels [56]. However, a recent randomized controlled trial conducted in children in Mongolia found that weekly supplementation of 14,000 IU of vitamin D<sub>3</sub> over 3 years did not lower risk of tuberculosis infection, tuberculosis disease, or other acute respiratory infection compared with placebo [57]. Among renal transplant recipients, those with vitamin D deficiency were found to have an increased risk of urinary tract, bacterial, or viral infections after renal transplantation [58–61].

## 3. Vitamin D deficiency prior to organ transplant

Vitamin D insufficiency and deficiency have been described in patients with end-stage pulmonary disease [62,63], liver failure [20], [64–68], congestive heart failure [69,70], and chronic kidney disease (CKD) [71–73]. Several factors place these patients at particular risk for vitamin D deficiency, including limited sunlight exposure and low dietary intake of vitamin D-containing foods. Furthermore, hepatic dysfunction, which can result from intrinsic liver disease or from hepatic congestion in heart failure patients, may contribute (Table 74.1). In this chapter, we will define insufficiency as 25(OH)D < 30 ng/mL, deficiency as 25(OH)D < 20 ng/mL, and severe deficiency as 25(OH)D < 10 ng/mL [74].

### 3.1 Vitamin D deficiency in patients with end-stage pulmonary disease

Patients with end-stage pulmonary disease commonly have 25(OH)D deficiency. Severe vitamin D deficiency has been reported in 20%–50% of these individuals [62,75,76]. In patients with advanced pulmonary disease, low 25(OH)D was associated with lower fat mass, obstructive pulmonary disease, and low dietary vitamin D intake and was a predictor of decreased walking distance and decreased forced expiratory

**TABLE 74.1** Risk factors for vitamin D deficiency in organ transplant patients.

- African American race
- Limited sunlight exposure, northern latitude, and winter months
- Low dietary intake of vitamin D
- Low fat mass
- Low serum albumin
- Hepatic dysfunction
- Obstructive pulmonary disease
- Renal insufficiency
- Diabetes
- Malabsorption
- Poor general health
- Female sex<sup>a</sup>
- Glucocorticoid use<sup>a</sup>—increases catabolism of 25(OH)D
- Organ transplanted<sup>a</sup>—liver-transplanted recipients may be at increased risk
- Recent transplantation<sup>a</sup>
- Proteinuria<sup>a</sup>
- Use of angiotensin-converting enzyme inhibitors or aldosterone receptor blockers<sup>a</sup>

<sup>a</sup>Risk factor specifically demonstrated following transplantation.

volume in 1 s ( $FEV_1$ ) [77,78]. In patients with cystic fibrosis (CF), a common indication for lung transplantation, we have observed that vitamin D deficiency is extremely common, related in part to pancreatic insufficiency. Despite supplementation, bone mineral density (BMD) was significantly lower in the D-deficient patients with CF [79]. Vitamin D deficiency is associated with osteoporosis and fractures in other cohorts with CF [79–81]. A recent study indicates that adults with CF and vitamin D deficiency are at higher risk of developing CF-related diabetes and are at risk for earlier onset of CF-related diabetes [82].

### 3.2 Vitamin D deficiency in patients with liver failure

Vitamin D deficiency is also very common in patients with severe liver disease, affecting over 80% of liver transplant candidates [66,83,84]. In one study of patients awaiting transplantation, mean 25(OH)D was 9 ng/mL, in the severely deficient range [64]. In a study of 107 liver transplant candidates, 84% were vitamin D deficient at

the time of liver transplant evaluation and 74% remained deficient at transplant [66]. In another cohort, cirrhotic patients referred for liver transplantation had lower serum 25(OH)D, 1,25(OH)<sub>2</sub>D, intact parathyroid hormone (PTH), and osteocalcin and higher urinary hydroxyproline excretion compared with controls [85]. PTH may have been low in the setting of vitamin D deficiency due to low intracellular magnesium, which can lead to reduced PTH release; however, this was not directly measured by the investigators. Also, it cannot be assumed that these patients had increased bone turnover based upon the elevated urinary hydroxyproline, because this marker can also be elevated in cirrhosis due to increased collagenolysis [85]. Another study found that a model for end-stage liver disease (MELD) score of greater than 15, indicative of worse disease and poorer health, was associated with lower serum 25(OH)D [86]. It is conceivable that free vitamin D may not be as low as suggested by total serum 25(OH)D measurements in patients with severe liver disease, who may have lower levels of vitamin D-binding protein (DBP) as a result of reduced hepatic synthetic capacity.

### 3.3 Vitamin D deficiency in patients with congestive heart failure

Vitamin D deficiency is common among patients with heart failure [87]. We reported that the majority of patients with congestive heart failure had vitamin D deficiency (mean 25(OH)D 18 ng/mL) and that 18% of patients had severe deficiency (<9 ng/mL). Patients with lower 25(OH)D had lower serum calcium, phosphorus, and albumin, and higher total alkaline phosphatase activity, and bone resorption markers. No association between 25(OH)D and 1,25(OH)<sub>2</sub>D was found. Serum PTH was significantly lower in those patients in the highest tertile of 25(OH)D [69]. In an observational study of patients with end-stage heart failure, lower circulating calcitriol levels were associated with poor clinical outcomes and death [88]. Vitamin D deficiency has been associated with left ventricular hypertrophy, vascular dysfunction, and activation of the renin-angiotensin system [89]. However, while vitamin D deficiency is associated with increased mortality and morbidity in patients in the aforementioned observational study, a recent randomized controlled trial found no significant improvement in blood pressure or 6-min walk test after administration of 50,000 IU of vitamin D<sub>3</sub>/week for 8 weeks [90]. Further, in a 3-year randomized clinical trial, supplementation of 4000 IU of vitamin D<sub>3</sub> daily did not reduce mortality in patients with advanced heart failure [91].



### 3.4 Vitamin D deficiency in patients with chronic kidney disease

In patients with CKD, glomerular loss leads to declining renal function and subsequent  $1,25(\text{OH})_2\text{D}$  deficiency [92,93]. Patients with CKD also frequently have  $25(\text{OH})\text{D}$  deficiency [71,72], [93–95]; lower  $25(\text{OH})\text{D}$  in this population is associated with worse kidney function, immune dysfunction, and possibly increased risk of infection [96–98]. A recent study indicates vitamin D may serve as a biomarker of tubular health and may be a predictor of worsening kidney function in stage 3 and 4 kidney disease patients [99]. In a population-based study, 71% of patients with stage 3 and 83% of those with stage 4 CKD had  $25(\text{OH})\text{D}$  insufficiency and 14% of patients with stage 3 and 26% with stage 4 CKD had severe deficiency [72]. In another study, 39% of CKD patients had  $25(\text{OH})\text{D}$  between 16 and 30 ng/mL, 33% less than 16 ng/mL, and 6% less than 5 ng/mL [71]. Factors associated with low  $25(\text{OH})\text{D}$  in CKD patients include female sex, African American race, latitude, season, diabetes, and low serum albumin [93,100]. Baseline  $25(\text{OH})\text{D}$  was an independent predictor of death over 6 years in patients with CKD [96]; this finding most likely reflects the poorer general health of the subjects with vitamin D deficiency at baseline.

## 4. Vitamin D deficiency following organ transplant

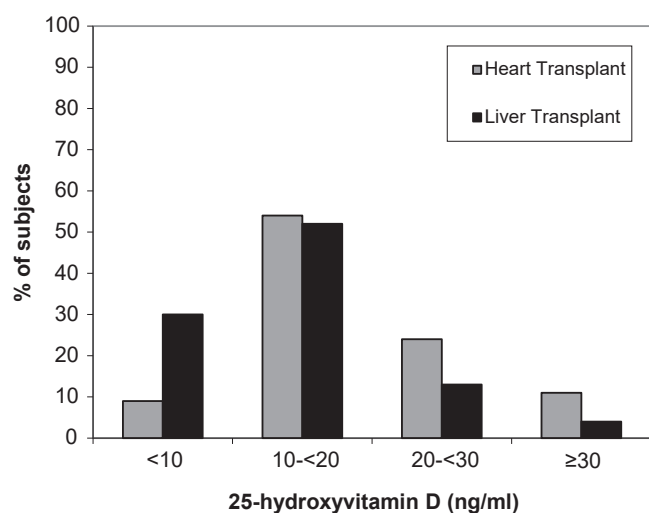
Vitamin D insufficiency has been reported in 51%–97% of organ transplant recipients and severe deficiency in 26%–33% [101–106]. Variability in these estimates relates to the type of organ transplanted, patient population, and assay utilized for measurement of  $25(\text{OH})\text{D}$  [107–110]. Poor health following transplant can lead to decreased dietary intake of vitamin D-containing foods. In addition, many organ transplant recipients dramatically limit sun exposure because they are at increased risk for skin cancer [111,112]. Furthermore, animal studies suggest that glucocorticoids, commonly used for posttransplant immunosuppression, may increase catabolism of  $25(\text{OH})\text{D}$  [113,114]. Patient factors that have been shown to be associated with vitamin D deficiency after transplantation include African American race [105,115], avoidance of sun, low dietary intake [104], and transplantation during winter months [115,116]. Variations in  $25(\text{OH})\text{D}$  related to sun exposure have not been observed in all studies [101], perhaps because sun exposure is so limited in the most severely ill patients.

### 4.1 Vitamin D deficiency at the time of organ transplantation

Few studies have examined vitamin D levels at the time of transplantation. One study found that 80% of recently transplanted lung transplant recipients were  $25(\text{OH})\text{D}$  deficient [117] and that low  $25(\text{OH})\text{D}$  levels measured during the 100 days immediately before or after lung transplant were associated with increased incidence of both acute rejection and infection in both the immediate posttransplant period and up to 1 year later [117]. In pediatric liver transplant patients, vitamin D insufficiency prior to transplant was associated with posttransplant development of T cell–mediated rejection [20]. In heart and liver transplant recipients evaluated immediately after transplantation, we found that 91% of patients had serum vitamin D insufficiency ( $25(\text{OH})\text{D}$  levels <30 ng/mL), 55% had deficiency ( $25(\text{OH})\text{D}$  <20 ng/mL), and 16% had severe deficiency ( $25(\text{OH})\text{D}$  levels <10 ng/mL). Vitamin D levels were significantly lower in liver compared with heart transplant recipients (Fig. 74.1), likely related to malabsorption and impaired hepatic  $25$ -hydroxylation of vitamin D in liver transplant patients. Lower levels of vitamin D-binding protein (DBP) may also contribute to lower  $25(\text{OH})\text{D}$  in liver transplant recipients, although we did not measure DBP in that study [101]. Vitamin D insufficiency was found in 59% of patients at the time of renal transplantation, with severe deficiency in 29% [115].

### 4.2 Vitamin D deficiency in long-term transplant recipients

In kidney transplant patients, vitamin D deficiency is common and severe several years after transplantation [102,105], [118–120]. In one cohort, 97% of renal transplant recipients had insufficient  $25(\text{OH})\text{D}$  levels. Although not directly associated with vertebral fractures, low  $25(\text{OH})\text{D}$  was associated with higher PTH, which was significantly associated with vertebral fractures [121]. In another study of renal transplant recipients who were on average 7 years posttransplant, serum  $25(\text{OH})\text{D}$  levels were lower than in age-matched controls, mean serum  $25(\text{OH})\text{D}$  was 10 ng/mL, and one-third of patients had undetectable levels [102]. In a study of 293 kidney transplant recipients, low  $25(\text{OH})\text{D}$  levels predicted both decline in glomerular filtration rate and requirement for glucocorticoids to treat allograft rejection within the first 10 years after kidney transplantation [122]. In 435 renal transplant patients, who were followed for up to 7.5 years from transplant, there was an inverse association of  $25(\text{OH})\text{D}$  levels with mortality, even after adjustment for potential cofounders such as



**FIGURE 74.1** Comparison of serum 25-hydroxyvitamin D levels at the time of organ transplantation in heart and liver transplant recipients. Adapted from Ref. [101].

renal function (HR 0.68, 95% CI 0.52–0.89) [123]. Additionally, renal transplant patients with endothelial dysfunction, a marker of cardiovascular health and mortality, had significantly lower vitamin D levels than those with normal endothelial function [124]. In more recent studies, vitamin D deficiency is still common, but the prevalence is lower, possibly because of greater awareness and treatment of vitamin D deficiency [125].

Factors associated with low 25(OH)D in renal transplant recipients include African American race [105], female sex, measurement in autumn and winter months [105], recency of transplantation [118], inadequate dietary vitamin D intake (reported in 87%–91% of patients [126]), proteinuria [127], and use of angiotensin-converting enzyme inhibitors or aldosterone receptor blockers [118]. Persistent elevations in fibroblast growth factor 23 (FGF23) after kidney transplantation may result in lower serum 1,25(OH)<sub>2</sub>D levels through multiple pathways. High FGF23 levels after renal transplant may contribute to PTH resistance [128,129].

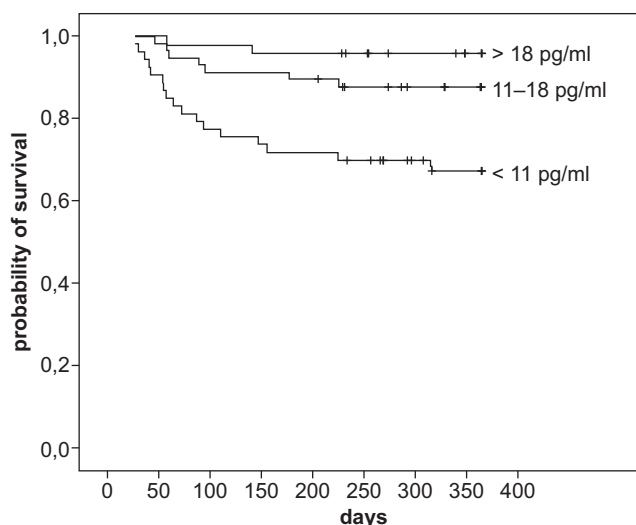
The optimal amount of vitamin D that is both beneficial and safe in transplant recipients remains to be determined, as does the target serum 25(OH)D level [130]. Data to address this knowledge gap may be available in the future through the vitamin D supplementation in renal transplant recipients trial, which is a prospective, multicenter, randomized trial of the efficacy and safety of cholecalciferol 100,000 IU monthly to maintain serum levels of 25(OH)D between 30 and 80 ng/mL [131].

Serum 25(OH)D concentrations are low in liver transplant recipients, likely because of disease-related factors such as malabsorption, impaired hepatic 25-hydroxylation of vitamin D, and reduced production of DBP [101,132]. In long-term liver transplant

recipients, 65%–68% had 25(OH)D levels below 15 ng/mL [103,133]. Patients with lower 25(OH)D levels also had lower Z-scores at the FN [133]. Many authors report increases in both 25(OH)D and PTH following liver transplantation [64,65,134,135]. However, others have not found significant changes [136–138]. Reported increases appear to be sustained for at least 3–4 years following transplantation [64,134]. In a recent observational study, lower baseline, preoperative 25(OH)D was associated with greater severity of liver disease. Moreover, lower serum 25(OH)D on postoperative day 28 was associated with incomplete graft recovery [132]. In an observational study of liver transplant patients, those supplemented with cholecalciferol had fewer rejection episodes [86].

In contrast, in a randomized placebo controlled trial, once monthly oral vitamin D supplementation of 100,000 IU after lung transplantation was not associated with chronic lung allograft dysfunction, lymphocytic bronchiolitis, respiratory infections, or pulmonary and systemic inflammation [139]. Whether smaller, but more frequent doses would have a different effect is an important question for future studies.

In cardiac transplant patients, vitamin D deficiency is frequently reported [69,140,141]. Furthermore, low concentrations of 1,25(OH)<sub>2</sub>D measured 21 days posttransplantation were directly associated with 1-year mortality (Fig. 74.2) [142]. After cardiac transplantation, sustained increases in serum creatinine [143–145] and decreases in 1,25(OH)<sub>2</sub>D are observed [142,144,145]. Whether this observation reflects the possibility that low calcitriol is a marker of renal dysfunction or poorer health or alternatively reflects a causal relationship



**FIGURE 74.2** Kaplan–Meier survival estimates in cardiac transplant recipients according to categories of serum calcitriol concentrations 21 days after transplant (log-rank test  $P < .001$ ). Adapted from Ref. [142].

between calcitriol and mortality requires further investigation. Factors associated with vitamin D deficiency in transplant recipients are detailed in [Table 74.1](#).

### 5. Treatment of posttransplant bone loss with vitamin D and analogs

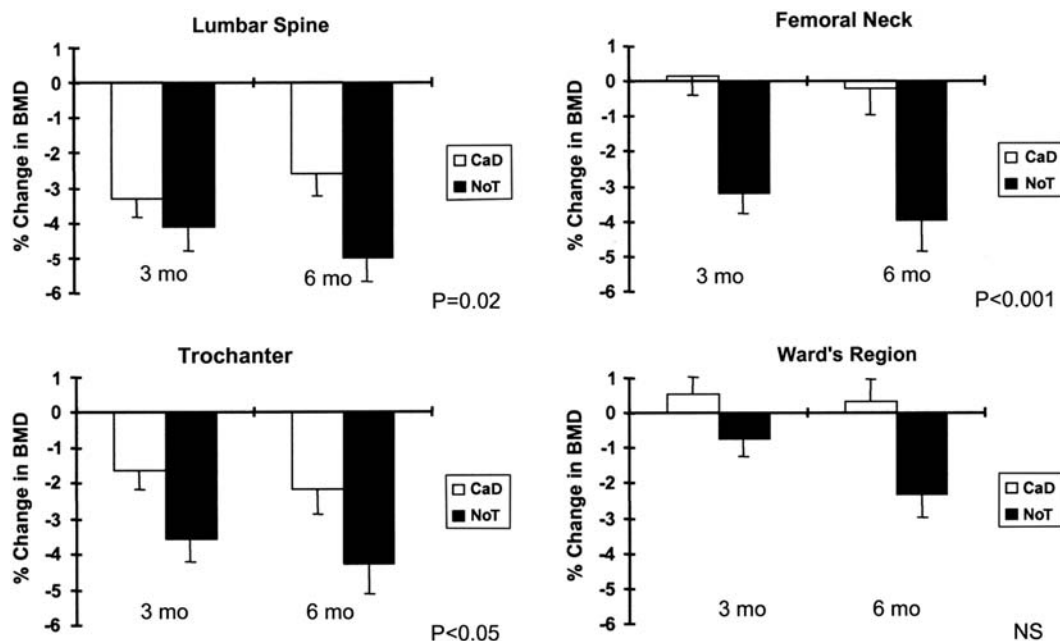
Vitamin D and its analogs have been used to prevent or treat osteoporosis after transplantation [146]. These agents may influence posttransplantation bone loss through several mechanisms. They may overcome glucocorticoid-induced decreases in intestinal calcium absorption, reduce secondary hyperparathyroidism, promote differentiation of osteoblast precursors into mature cells, or influence the immune system and potentiate the immunosuppressive action of calcineurin inhibitors and prednisone, thus reducing the required dose of these drugs [24,98,147,148].

The majority of older observational studies that documented high rates of bone loss after organ transplantation included at least 400–800 IU of parent vitamin D in the posttransplant regimen; thus, it is clear that the recommended daily allowance for vitamin D is not sufficient to prevent posttransplantation bone loss. In two studies, parent vitamin D, at doses of 800 IU daily [149] or 25,000 IU monthly [150], did not prevent bone loss after kidney transplantation. In a recent observational study, it was reported that supplementation with 25(OH)D in doses of 25,000 IU/week for 12 weeks followed by 1500 IU/day for 2 years was not associated with changes in BMD over 2 years in kidney transplant

recipients [151]. However, in view of data summarized earlier in the chapter suggesting that adequacy of vitamin D or therapy with vitamin D is associated with better immune function, a number of organizations including the International Society for Heart and Lung Transplantation, American Association for the Study of Liver Disease, and the Kidney Disease Improving Global Outcomes recommend 400–1000 IU of vitamin D daily for recipients of solid organ transplants [152–155]. In a recent randomized control trial, 4000 IU of daily vitamin D (cholecalciferol) attenuated spine bone loss in renal transplant recipients [156].

More active forms of vitamin D may have greater efficacy than simple vitamin D. Calcidiol (25(OH)D) prevented bone loss and increased LS BMD after cardiac transplantation [157]. When administered immediately after kidney transplantation, alfacalcidol (1 $\alpha$ -OHD) prevented or attenuated bone loss at the LS and FN [158–161]. De Sevaux and colleagues [160] found that alfacalcidol treatment during the first 6 months following renal transplant attenuated bone loss at the LS and greater trochanter and prevented loss at the FN ([Fig. 74.3](#)). Several studies have found increased BMD at the lumbar spine (LS) and femoral neck (FN) in renal transplant patients receiving activated vitamin D (calcitriol and alfacalcidol) [131,158]. Barros et al. found that calcifediol (calcidiol) administered either monthly or biweekly at a 266 mcg/dose safely and effectively lowered PTH and raised 25(OH)D levels in 168 renal transplant patients with a functioning allograft for more than 6 months [162].

In kidney transplant patients, even though there is an increase in calcitriol production by the transplanted

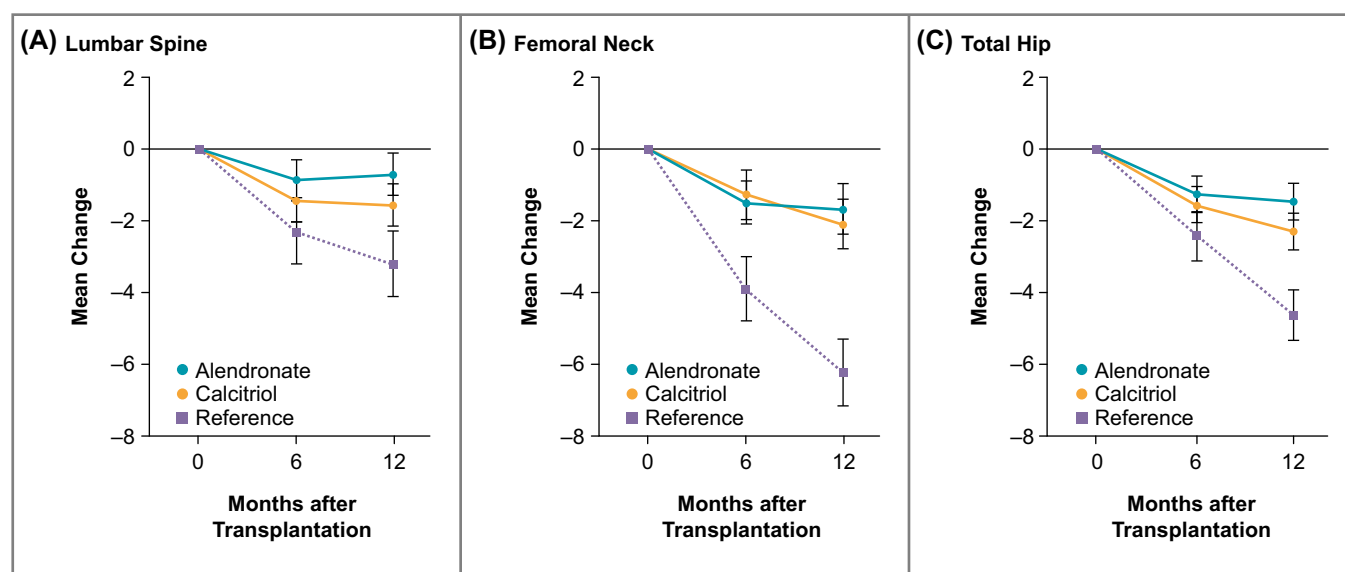


**FIGURE 74.3** Change in bone mineral density in renal transplant recipients treated with calcitriol or placebo (% from baseline  $\pm$  SE; significance shown for differences between groups at 6 months). Adapted from Ref. [160].

kidney, it may be inadequate to suppress excess PTH secretion by hyperplastic parathyroid glands [163]. Calcitriol treatment may be of particular benefit in these patients [22]. Treatment with calcitriol may prevent hyperparathyroidism after cardiac transplant as well [25]. Some studies have found beneficial effects on BMD at doses greater than 0.5  $\mu\text{g}$  per day, although this finding is not uniform. Calcitriol given during the first year after kidney transplantation was associated with an increase in lumbar spine (LS), femoral neck (FN), and forearm BMD [164]. In another study of renal transplant recipients, intermittent calcitriol and calcium prevented bone loss at the total hip but not LS [165]. In a stratified, placebo-controlled randomized study, in which heart and lung transplant recipients received calcitriol or placebo for 12 or 24 months after transplantation [166], LS bone loss was minimal and did not differ between groups. At the FN, bone loss at 24 months was significantly reduced but only in the group that received calcitriol for the entire period. In contrast to these results, which suggest that the protective effects of calcitriol are not sustained after cessation of treatment, we found no bone loss at either the spine or hip in cardiac transplant patients when calcitriol was discontinued after the first posttransplant year [167]. In contrast to the aforementioned findings, other studies have failed to find any benefit of calcitriol on BMD after transplant [168–170].

Vitamin D analogs have been compared with bisphosphonates in several studies, with mixed results. In a randomized trial, we found that the bisphosphonate alendronate (10 mg daily) or calcitriol (0.25  $\mu\text{g}$  twice daily) given immediately after cardiac transplant provided similar protection against bone loss at the spine

and hip 1 year after transplant (Fig. 74.4) and that both were superior compared with a reference group receiving only calcium and vitamin D [25]. During the second year after cardiac transplant, BMD remained stable after discontinuation of both drugs [167]. During the first 6 months after heart or lung transplantation, calcitriol prevented bone loss at the spine and hip and was as effective as cyclic etidronate [171]. In liver transplant patients, administration of ibandronate in addition to calcitriol 0.25–1.0  $\mu\text{g}$  daily, calcium 1000 mg daily, and cholecalciferol 800 IU daily led to increases in FN BMD that were maintained throughout the 3-year study period; in contrast, the control group who received calcitriol, calcium, and vitamin D alone experienced decreases in FN BMD [172]. Kidney transplant patients treated with alendronate, calcitriol, and calcium had increases in LS BMD compared with decreases in those who received only calcium and calcitriol [173]. In another trial of long-term kidney-transplanted patients, approximately 5 years after transplantation who were started on either alendronate, calcitriol, and calcium or only calcitriol and calcium, those in the alendronate, calcitriol, and calcium group had significant improvements in spine and hip BMD, while BMD in the calcitriol and calcium group was stable [174]. In a randomized trial of long-term kidney-transplanted recipients that compared alendronate alone to alfacalcidol plus alendronate for 1 year, BMD improved at the spine and hip in both groups. However, the increase was only significant in the combination alendronate–alfacalcidol group, likely because of inadequate power in this small study [175]. While the results of the aforementioned studies are not uniform, the majority found that active vitamin D analogs when given alone or in combination with



**FIGURE 74.4** Comparison of mean ( $\pm$ SE) percent change in bone mineral density from baseline in cardiac transplant subjects treated with alendronate or calcitriol and untreated reference group. Adapted from Ref. [25].



bisphosphonates were effective for maintaining or improving BMD after transplant.

The major side effects of therapy with active vitamin D and analogs are hypercalcemia and hypercalciuria, which may develop at any time during the course of treatment, necessitating frequent urinary and serum monitoring. Additionally, concern has been raised about using very high single annual dose vitamin D (500,000 IU annually) and its impact on morbidity in terms of falls and fractures in the elderly [176]. Given the narrow therapeutic window with respect to hypercalcemia and hypercalciuria and monitoring requirements as well as the demonstrated efficacy of bisphosphonates to prevent posttransplantation bone loss [25,177], we believe these vitamin D analogs should be adjunctive rather than primary therapy for the prevention and treatment of transplantation osteoporosis.

It is important to test for and correct vitamin D deficiency before treating transplant candidates or recipients with IV bisphosphonates or denosumab. Bisphosphonates may not be optimally effective in the setting of vitamin D deficiency. Furthermore, intravenous bisphosphonate treatment has been reported to precipitate symptomatic hypocalcemia in patients with severe, unrecognized vitamin D deficiency [178]. Denosumab, a monoclonal antibody inhibiting RANK-L, blocks osteoclast activation and is commonly used to treat osteoporosis. Denosumab has shown great promise in reduction of both hip and nonvertebral fractures in postmenopausal women and BMD preservation in people with rheumatoid arthritis on high-dose glucocorticoids [155] but has not been widely studied in the solid organ transplant population. In one study of renal transplant recipients, denosumab increased BMD at the spine and hip when given every 6 months in the first year following transplant [179]. Analysis of microarchitectural changes in these patients using high-resolution peripheral quantitative CT (HR-pQCT) showed that there were increases in cortical thickness, bone stiffness, and failure load at the tibia in those randomized to denosumab, suggesting that both bone quality and BMD are improved with denosumab use in kidney transplant recipients at risk for osteoporosis [180]. A recent systematic review of four observational studies and the aforementioned clinical trial similarly reported increased BMD at the spine and hip with denosumab treatment [181]. Denosumab treatment is also associated with hypocalcemia in patients with severe vitamin D deficiency and inadequate calcium intake, and also in those with renal insufficiency despite calcium and vitamin D supplementation [180]. Denosumab also may be associated with increased infection risk, making it a potentially less ideal choice in those who are awaiting organ transplant and potentially already immunocompromised [155].

## 6. Conclusions

Vitamin D deficiency is extremely prevalent in patients with organ failure before transplantation and among transplant recipients. All patients should be assessed for vitamin D insufficiency and deficiency before transplantation and, if present, receive treatment. In addition, long-term transplant recipients should be monitored and treated for vitamin D deficiency in the context of broader management of bone disease. Pharmacologic doses of vitamin D and its analogs have utility after renal transplant but should be considered as adjunctive rather than primary therapy for osteoporosis in patients after solid organ transplantation. Treatment of vitamin D deficiency may improve skeletal health in transplant patients. The effects of vitamin D deficiency and in particular vitamin D supplementation on extra-skeletal morbidity after transplant are less clear. Additional interventional studies are needed to elucidate optimal repletion regimens after transplantation and to determine whether restoring 25(OH)D at the time of transplant reduces the development of infectious complications and immunosuppressant requirements.

## 7. Summary points

- Vitamin D plays a role in the regulation of immune cell proliferation, differentiation, and responsiveness; some evidence suggests a role of 1,25(OH)<sub>2</sub>D in prevention of graft rejection and opportunistic infections after transplantation.
- Vitamin D insufficiency and deficiency have been commonly described in patients with end-stage lung, liver, heart, and kidney disease prior to organ transplant.
- Vitamin D and its analogs may mitigate posttransplantation bone loss through several mechanisms including overcoming glucocorticoid-induced decreases in intestinal calcium absorption, reducing secondary hyperparathyroidism, and promoting differentiation of osteoblast precursors into mature cells.
- While the results of the current literature are not uniform, the majority of studies have found that active vitamin D analogs, given alone or in combination with bisphosphonates, maintain or improve BMD after transplant.

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# Vitamin D, obesity, the metabolic syndrome and its sequelae

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## OBJECTIVES

- To review evidence for lower vitamin D concentrations in obesity and to discuss how related differences arise.
- To review evidence for the efficacy of vitamin D supplementation in obesity and the implications of obesity for vitamin D requirement.
- To discuss obesity-related differences in vitamin D concentrations and the implications this can have on metabolic syndrome, related disorders, and other diseases.
- To consider the possible role of vitamin D on the risk of obesity.

## 1. Introduction

There is continuing interest in the role of adipose tissue in the determination of vitamin D status (serum 25-hydroxyvitamin D [25(OH)D] concentration) and of both vitamin D requirements and its bioavailability, because measures of human adiposity consistently relate inversely to serum 25(OH)D. Indeed, obesity was second only to low sun exposure as a predictor of the severity of vitamin D insufficiency (low serum 25(OH)D) in an earlier study [1,2]. The various mechanisms now thought to explain the increased risk of vitamin D deficiency found with obesity include both the dilution of vitamin D and of 25(OH)D into the

enlarged fat pool and the fact that the 25-hydroxylation of vitamin D by the liver has been shown to be reduced in obesity [3,4]. There is substantial variation in the estimated strength of this association among different population groups, which probably reflects methodological issues in the measurement of adiposity and the variations reported in measurements of 25(OH)D concentrations [5]. Also, similar body mass indices (BMIs) are found in different population groups despite markedly different ratios of visceral to subcutaneous fat deposition [6,7]. Such variations are important in assessing health risks as visceral adiposity is known to be a stronger risk factor than subcutaneous adiposity for the metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), and cardiovascular disease (CVD) [8,9].

Active hormonal vitamin D [1,25(OH)<sub>2</sub>D] has been shown, at both physiological and biochemical levels, to have mechanistic effects that counteract many ill effects of obesity and that reduce the risks of tissue damage seen with adiposity and in obesity-related metabolic disorders. Those data imply that ensuring adequate vitamin D nutrition in obesity should provide some protection against the adverse sequelae of obesity, including the various features of the MetS, which are more common in overweight and obese people than in slim people. This chapter, therefore, includes a brief overview of the demonstrable impacts of vitamin D on the disorders that comprise the metabolic syndrome and its sequelae and on inflammation, including the remote inflammatory tissue damage and increased cell senescence associated with obesity, especially relevant to atheromatous disease [10,11].



## 2. Adipose tissue as a vitamin D reserve

Adipose tissue was first proposed as a major storage site for vitamin D by Rosenstreich et al. [12]. This proposition was based on the fact that vitamin D is a fat-soluble vitamin, and on the measurements of tracer radioactivity contained in different vitamin D metabolites within the tissues and organs of weanling rats after they had been fed radiolabeled vitamin D<sub>3</sub> for 2 weeks. Vitamin D was found to be stored in its various forms, but intact (unaltered) vitamin D<sub>3</sub> was the principal form stored [12]. About 75% of intact vitamin D<sub>3</sub> in the human body is found in fat. For 25(OH)D, ~30% is present in the circulation, 20% in skeletal muscle, and 34% in adipose tissue [13]. Interestingly, adipose tissue is itself a site of vitamin D metabolism where both 25-hydroxylation and further 1 $\alpha$ -hydroxylation of 25(OH)D (via the enzyme 1 $\alpha$ -hydroxylase or CYP27B1) to form hormonally active 1,25(OH)<sub>2</sub>D take place, as does homeostatic catabolism of those metabolites via the enzyme 24-hydroxylase.

The amount of intact vitamin D in adipose tissue does not correlate with circulating cholecalciferol or 25(OH)D concentrations. Serum 25(OH)D concentrations do, however, correlate directly with those seen in both visceral and subcutaneous fat [14–16]. Wortsman, therefore, suggested that vitamin D is “sequestered” in adipose tissue, intact vitamin D passing into adipose tissue and being prevented from being further processed or passing back into the circulation [17]; a proposal based on finding of notably greater elevations of intact serum vitamin D<sub>3</sub> concentrations in normal than in obese people after exposure to ultraviolet B radiation [17]. However, another explanation is that serum 25(OH)D is lower in obesity because this metabolite simply spreads across the larger volumes of fat (volumetric dilution). This suggestion is supported by the fact that serum and adipose tissue 25(OH)D concentrations, but not cholecalciferol concentrations, are correlated across the life span. CYP27B1 concentration is lower in the white adipose tissue of obese versus slim people, likely reducing local vitamin D activation [18]. Differences in behaviors affecting vitamin D nutrition (such as habitual sun exposure, time spent outdoors, and choice of clothing) may also influence the contributions to serum 25(OH)D derived from the vitamin D synthesized in the skin in obese as compared with normal-weight individuals, though it is still unclear how vitamin D metabolites are handled once taken up by the adipose tissue.

1 $\alpha$ -Hydroxylase is present and active in adipocytes [19], but the “efficacy” of orally consumed vitamin D is reduced in obesity (i.e., it does not raise serum 25(OH)D concentrations as much). In addition, “deep” subcutaneous adipose tissue from obese subjects

contains less of both the vitamin D-activating enzymes than does “deep” subcutaneous fat of normal-weight subjects (by –71% for the vitamin D 25-hydroxylase and by –49% for 1 $\alpha$ -hydroxylase). Though the adipose tissue concentrations of both intact and 25-hydroxylated vitamin D are lower in fat from obese than from lean women, their 1,25(OH)<sub>2</sub>D concentrations are not different [20]. However, since serum 1,25(OH)<sub>2</sub>D is reduced in obesity, it could be that significant activation of vitamin D in adipose tissue compensates for the reduced 25(OH)D substrate availability from the serum [20].

Visceral and subcutaneous adipose tissues are not only metabolically “active” for vitamin D, but they also express the vitamin D receptor (VDR) that mediates both rapid nongenomic and slower genomic effects of 1,25(OH)<sub>2</sub>D through cell wall and nuclear vitamin D receptors (VDRs), respectively [21] (see Chapters 10–13). In addition, both these adipose tissues express the 24-hydroxylase enzyme that is catabolic for both 25(OH)D and 1,25(OH)<sub>2</sub>D [18]. Overall, therefore, the combined evidence suggests that adipose tissue can both synthesize and degrade vitamin D metabolites, thereby facilitating autocrine and paracrine effects on adipogenesis, lipid metabolism, and the regulation of inflammatory responses associated with obesity [21]. For a fuller description of vitamin D influences on energy homeostasis in adipose tissue, please see Chapter 28.

In addition to local intracrine effects, paracrine roles of 1,25(OH)<sub>2</sub>D in adipose tissue could contribute to, or account for, loss of 25(OH)D into fat, although a simple “dilution” effect could also occur [2]. According to the initial proposal by Rosenstreich [12], release of vitamin D metabolites from adipose tissue would be relatively slow, reducing potentially toxic effects of high dosages, yet allowing adipose tissue to act as a store in case of need. Such a system would contribute to the homeostasis of vitamin D activity, but other systems regulating skin synthesis of vitamin D and renal activation are likely to be more powerful in health. However, in starvation this mechanism may be important for the provision of vitamin D because marked weight loss does increase serum 25(OH)D concentrations [22], in the same way that marked weight loss is known to release other fat-soluble substances known to accumulate in fat, e.g., various toxic insecticides including DDT (dichlorodiphenyltrichloroethane) [23,24].

It is still uncertain whether adipose tissue uptake of vitamin D or its metabolites depends on its conventional association with vitamin D-binding proteins (DBPs), although DBP does carry 25(OH)D into renal cells for local activation to form hormonal 1,25(OH)<sub>2</sub>D (see Chapter 8). DBP knockout in rodents potentiates, but does not reduce, 1,25(OH)<sub>2</sub>D formation, suggesting

that unbound (“free”) 25(OH)D is important as a substrate for local tissue activation. The question of whether DBP binding of vitamin D metabolites such as 25(OH)D is necessary for its uptake by adipose tissues is important because, if free circulating 1,25(OH)<sub>2</sub>D is taken up, it would have the potential to help protect adipose tissue function despite the reductions in total serum 25(OH)D concentrations that are seen in obesity (see Chapter 7).

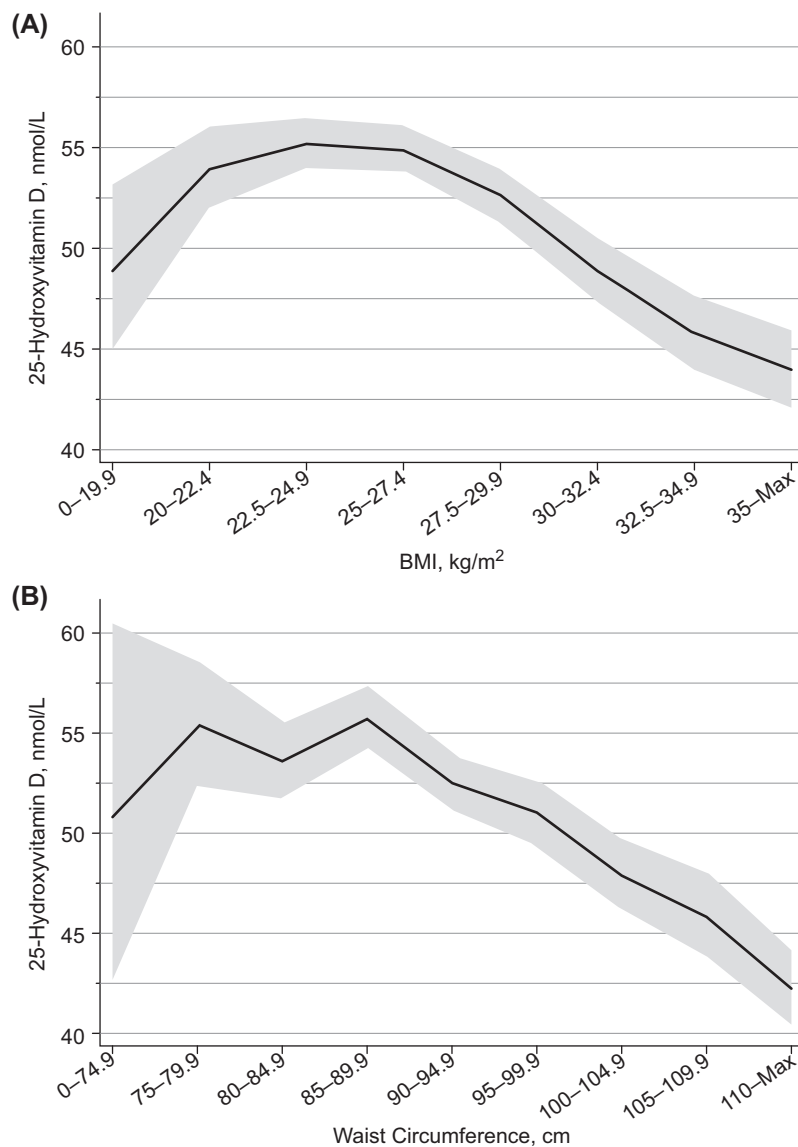
### 3. Obesity and vitamin D deficiency

It is well established from many metaanalyses that there is a higher prevalence of vitamin D deficiency (low serum concentrations of 25(OH)D) in those who are overweight or obese. Bidirectional Mendelian randomization (MR) data analysis has shown clearly that higher BMI leads to lower serum 25(OH)D concentrations but that linear reductions in 25(OH)D do not increase BMI [25]. Pereira-Santos et al. reviewed 23 studies and found a 35% increase in the prevalence of vitamin D deficiency for those classified as obese versus those of normal weight [26]. Similar rates of vitamin D deficiency in obesity were reported in children and adolescents (37%) as in adults (33%) [26]. Yao and colleagues [27] found 3.4 times greater odds of vitamin D deficiency for obese individuals compared with nonobese subjects, with similar findings reported by others [28,29]. However, with common outcomes, odds ratios tend to be inflated as compared with prevalence ratios and the metaanalyses mentioned before included studies with nonrandom population sampling, which often gave extremely high estimates; 5 of the 15 studies included in one metaanalysis having odds ratios >6, suggesting that estimates of effect size need review. Common to all metaanalyses assessing cross-sectional associations between obesity and vitamin D deficiency was considerable heterogeneity between the studies included. Furthermore, many used BMI as the primary indicator for adiposity, which, as already mentioned, is not a reliable measure of visceral adiposity. In addition, the associations between BMI and 25(OH)D are usually reported as being stronger in women than in men, probably reflecting gender differences in body composition as well as the inadequacy of BMI for assessing adiposity rather than any specific gender-related disorder of vitamin D metabolism in obesity. However, a small trial in overweight and prediabetic south Asian women supplemented at 60,000 IU weekly in milk for 8 weeks and followed for over a year showed reductions in truncal obesity with minimal reductions in glycemia [30]. The possibility that adipose tissue responses to vitamin D vary with ethnicity and/or gender should, therefore, be considered.

Despite some variations in their strength, inverse adiposity-25(OH)D associations have consistently been reported from studies in diverse age groups and from diverse geographical locations. These associations also appear to be present from infancy [31–33]. For example, higher circulating 25(OH)D values were reported with leaner body composition in 132 normal-term, nonobese, and breastfed babies aged from birth to 1 year old [31]. Correlations between adiposity and 25(OH)D have been reported at different ages and in populations of European, Asian, and African ancestry [26,32–34]. Geographical differences are also reported for the strength of those associations (e.g., stronger correlations between BMI and 25(OH)D for North Americans compared with European, and for Western as compared with Eastern countries [30,34,35]), which may reflect differences in the prevalence of obesity, and/or ethnic variations in fat distribution.

Another potential factor causing differences in the strength of the associations between adiposity and 25(OH)D is the prevalence rate for vitamin D deficiency. In the US National Health and Nutrition Examination Survey, for example, percentage body fat was more weakly associated with 25(OH)D concentrations in African Americans than in white American women [35], the authors suggesting this could reflect ethnic differences in adiposity–25(OH)D relationships, or the lower 25(OH)D concentrations in black versus white people that provide smaller reserves of this metabolite for distribution into adipose tissue. However, in that study body fat was measured by bioimpedance, which shows noticeable variations in precision of the estimates made for the different population groups [36], which could have contributed to the apparent differences. Such differences in association strength between studies or population groups may also reflect the lack of adjustment for the nonlinearity of these associations [37,38]. For example, in the 1958 British birth cohort (1958 BCE), a sample of white European-ancestry volunteers aged 45 years, the highest serum 25(OH)D concentrations were found with those of normal weight, with linear decreases with increasing overweight and obesity [38]. However, lower concentrations were also seen in participants who were notably underweight, which in this type of cross-sectional study may well have reflected the influence of reverse causality due to disease-related differences in vitamin D–relevant lifestyles, or requirements. Also, the similar nonlinear variations in 25(OH)D concentrations with adiposity that were found in the 1958 BCE whether adiposity was assessed using BMI or by waist circumference support the validity of this association [38] (Fig. 75.1).

The potential differences in 25(OH)D storage between different fat deposits remains unknown, but



**FIGURE 75.1** Variation in average serum 25(OH)D concentration by (A) body mass index (BMI) and (B) waist circumference in the 1958 British birth cohort (aged 45 year). Values are geometric means (95% confidence intervals) standardized by sex and season. Reproduced from Ref. [192] under the Creative Commons Attribution License.

observational data on associations between visceral/subcutaneous fat masses and serum 25(OH)D concentrations have shown that both central and visceral fat increases were associated with reductions in 25(OH)D in obese/overweight middle-aged adults but not with the increases in peripheral fat [39]. Andreozzi et al. found inverse correlations of waist circumference, and of waist/hip ratio with serum 25(OH)D [40]. A study in younger people (aged 6–18 years) also found adiposity associated inversely with serum 25(OH)D but showed no variation in that association with the different types of fat distribution [31], while studies continue to confirm visceral (central) adiposity as a better risk marker for

reduced vitamin D status than subcutaneous adiposity in adults [41].

#### 4. Obesity, DBP, and “free” vitamin D concentrations

The lower 25(OH)D concentrations seen in obesity imply “functional” vitamin D deficiency since it reduces 25(OH)D availability to target tissues. However, 99% of circulating 25(OH)D and of 1,25(OH)<sub>2</sub>D are bound to DBP or albumin (see Chapter 7). As with other hormone-binding proteins [42]. DBP concentrations

may, therefore, provide a reservoir of free 25(OH)D helping to regulate 25(OH)D bioavailability to target tissues and mean that the hormonal provision of 1,25(OH)<sub>2</sub>D within the target cells might be better supported by “free” 25(OH)D than by total 25(OH)D. However, until intracellular vitamin D metabolites can be quantified in cells *in vivo*, this remains in doubt. Renal and placental tissue access bound 25(OH)D by endocytic absorption of DBP through the actions of megalin and cubulin, not found in most other tissues [43]. Like total 25(OH)D, free serum 25(OH)D is inversely associated with weight in obesity. Vitamin D repletion has been suggested as a means of correcting, or at least improving, many different disorders, including the metabolic disorders associated with obesity where achieving repletion may require varying supplemental doses [44,45]. Some [46,47] but not all [48,49] studies suggest positive correlations between adiposity and DBP levels, and some studies also report reduced “free vitamin D” [free 25(OH)D] concentrations in obesity [46–50]. However, obesity’s effects on “free vitamin D” and whether free 25(OH)D affects bone health, or other health outcomes, remains in doubt.

## 5. The effect of weight loss on serum 25(OH)D concentrations

Since serum 25(OH)D values are lowered with increased adiposity, it is no surprise that weight loss, whether from dieting, or gastric banding, leads to rises in serum 25(OH)D [51,52]. Weight loss induces rises in serum 25(OH)D without increased expression of adipose 25-hydroxylase [2,18], making increases in adipose 25-hydroxyvitamin D production unlikely. Reversal of the hepatic reductions in 25(OH)D formation induced by obesity is a more likely explanation, though adipose CYP24A1 activity actually increases with weight loss [18] and reduced dilution into fat will also contribute. More recent NHANES data showed that weight loss of 5% over 5 or 10 years led to 25(OH)D rises of 5.5 and 5.1 nmol/L, respectively, in >6000 subjects [51]. In the heaviest people, serum 25(OH)D values were higher initially than in others, but concentrations did not rise with 5% or 10% weight loss, suggesting that intake levels may have been highest in the heaviest people. Despite the release of vitamin D and 25(OH)D into the circulation with weight reduction, this has not been reported to lead to toxic effects, no doubt because normal regulatory mechanisms continue to limit 1,25(OH)<sub>2</sub>D production (see Chapter 8 and 9). Additionally, there is longitudinal data to suggest that long-term changes in BMI predict changes in 25(OH)D concentration, so that vitamin D deficiency rates will fall in populations that achieve reductions in the prevalence of obesity, as was

seen prospectively in a subsample from the large Tromsø study [39], where participants with at least a one-unit reduction in BMI during the 14 years of follow-up averaged a 4.5 nmol/L increase in 25(OH)D concentration while those who gained weight averaged a reduction in serum 25(OH)D of –2.5 nmol/L. Some support for increases in 25(OH)D with weight loss comes from a systematic review of weight reduction trials in obesity [2,22]. One metaanalysis showed variable increases in serum 25(OH)D in 18 of 23 included trials, while metaregression analysis suggested an increase of 6 nmol/L in serum 25(OH)D for each 10 kg weight loss, while each 10% loss of fat mass was followed by an increase in serum 25(OH)D of 9 nmol/L [2]. Such findings have been supported by other similar studies though the changes were small and showed no suggestion of a dose–response relation [51].

It is likely that the quantity of 25(OH)D “released” with weight loss will vary with the proportion of fat loss from each type of fat store. Visceral adipose tissue regularly contains ~20% more cholecalciferol than subcutaneous fat so that reductions in visceral adiposity should increase serum 25(OH)D concentrations more than reductions in peripheral adiposity. In one lifestyle intervention trial in viscerally obese men, decreases in intraabdominal fat mass did have the highest impact on 25(OH)D outcomes, a 50% reduction in visceral fat being accompanied by a 27% rise in serum 25(OH)D concentrations [51]. That study further suggested that central fat loss achieved those increases in serum 25(OH)D concentrations in the absence of any changes in dietary vitamin D intake or in the use of vitamin D supplements. Studies of weight reduction through surgical intervention often include patients with quite low baseline 25(OH)D concentrations [52–54]. Major bariatric surgery, e.g., with gut bypass, often leads to long-term fat malabsorption, making correction of vitamin D deficiency difficult and the routine management of patients after bariatric surgery includes long-term vitamin D supplementation, though achieving 25(OH)D concentrations at or above 75 nmol/L is often difficult [54,55]. Current guidance from the European Calcified Tissue Society (ECTS) states that postbariatric surgery patients should be adequately provided with vitamin D before other bone-protective measures are introduced, that this advice should be kept under review, and that adjusting vitamin D intakes to individual need in such patients should be included in routine clinical management [56].

## 6. Can vitamin D supplementation prevent, or reduce, obesity?

Evidence available to date suggests that 1,25(OH)<sub>2</sub>D production in human adipose tissue is likely to be



homeostatically regulated, as it is in other target tissues, independent of parathyroid hormone. In addition, adipocyte maturation from preadipocytes is inhibited by  $1,25(\text{OH})_2\text{D}$  [57]. Thus, vitamin D adequacy should help to inhibit the enlargement of developing fat masses in early life, though it is not thought likely to reduce adult fat masses. In utero epigenetic effects resulting from lack of maternal vitamin D are, however, associated with increased obesity in childhood [58]. At a physiological level, there is evidence to suggest that the specific effects of  $1,25(\text{OH})_2\text{D}$  on adipose tissue could be protective for obesity because it increases lipolysis, reduces the lipid content of differentiated 3T3-L1' adipocytes, and reduces expression and activity of adipogenic genes while increasing expression of lipolytic genes and of NAD-SIRT-1 pathway activity [59–61], these effects being suggestive of a calorie-consuming response. Though obesity may not affect calcium-sensing receptor gene expression in white adipose tissue [59–61], vitamin D increases mature adipocyte apoptosis; this is affected through rapid nongenomic increases in intracellular calcium following cell wall caveolar VDR activation rather than resulting from activation of the nuclear VDR pathway [61] (see Chapters 10–13).

Despite such mechanistic evidence, RCTs assessing the effects of vitamin D supplementation have not provided convincing evidence of an effect of vitamin D on adiposity or adiposity indices in adult obesity. Some treatment effects have been reported, mainly from studies using vitamin D plus calcium. The largest of these studies is the Women's Health Initiative (WHI), which was a randomized double-blinded placebo-controlled trial performed in a cohort of 36,282 postmenopausal women that compared the daily dosage of 400 IU vitamin  $\text{D}_3$  (+1000 mg calcium) with placebo [62]. This WHI study reported a small reduction in weight gained during the 7-year trial of supplementation as compared with weight gained on the placebo, with an average difference of  $-130$  g. However, the evidence for differences in weight change was only seen in women who had baseline calcium intakes that were below the recommended dietary intake (DRI) of  $>1200$  mg/day, while no such effect was seen in women regularly consuming dietary calcium at, or above, the DRI for calcium [62]. Due to the design of that study, it was not possible to establish any definite independent effects of supplemental vitamin D from effects of supplemental calcium. However, given that the finding of reduced weight gain was dependent on baseline calcium intakes, the very small vitamin  $\text{D}_3$  dosages given, and that participants were also free to consume up to 1000 IU of vitamin D as part of their self-supplementation, it is unlikely that reduced weight gain resulted from the vitamin D component of the treatment regimen in that particular study.

Later, metaanalyses of available RCTs [63,64] also failed to provide evidence for weight reduction or changes in adiposity indices. However, these did allow for the evaluation of the effects of vitamin D supplementation,  $+/-$  = calcium versus placebo, and by vitamin D + calcium versus calcium alone. These metaanalyses identified no independent effects of vitamin D or evidence for a dose–response effects of supplemental vitamin D on adiposity [63] though some studies have suggested a reduction in visceral obesity [65].

Although the available evidence does not support any major effect of vitamin D supplementation on weight change or BMI in adults, there are still relatively few studies, which have investigated the effects of vitamin D supplementation on specific areas of fat tissue deposition in obesity. Potential benefits were suggested by two double-blind parallel trials providing vitamin D in fortified orange juice, one using regular orange juice and the other using low-calorie (50% reduced energy) orange juice to compare intakes of vitamin  $\text{D}_3$  (at 300 IU (7.5 mg))/day + calcium (1050 mg/day), against nonfortified juices [65]. Fortification led to some reduction in visceral adiposity in both trials but without changes in total body weight, BMI, or waist circumference. These last two trial results are of interest, but in no trial giving calcium and vitamin D can the effects of vitamin D be clearly disentangled from those of calcium. Genetic variations in the vitamin D axis may also affect responses to supplementation as suggested by an RCT in vitamin D–deficient subjects with T2DM, where central obesity was reduced most markedly in subjects with the VDR-Cdx-2 genotype [66].

The potential causal effect of higher serum  $25(\text{OH})\text{D}$  in regard to a lower BMI has also been investigated in genetic epidemiological studies, which have used genetic markers affecting substrate availability (*DHCR7*) or  $25(\text{OH})\text{D}$  synthesis (*CYP2R1*) to reflect differences in serum  $25(\text{OH})\text{D}$  concentrations [67]. However, rather than providing evidence in support of the causality of higher  $25(\text{OH})\text{D}$  concentrations as a determinant of lower BMI, these studies have consistently suggested that the observed correlations are driven by higher BMIs, leading to lower  $25(\text{OH})\text{D}$  concentrations [25,68]. One caveat with this type of genetic epidemiological, Mendelian randomization (MR), study, as well as with clinical trials of vitamin D supplementation conducted to date, relates to the lack of information specific for individuals who are clinically vitamin D deficient. Thus, even if vitamin D supplementation of general populations is not likely to influence weight regulation, from studies published to date, it is still not possible to fully exclude a possible causal influence on adipose tissue metabolism, or on obesity prevention, of corrective vitamin D prophylaxis in overtly vitamin D–deficient individuals [25], though this may be clarified if future

MR studies can use nonlinear analytical methodology (see Chapter 61 for further information).

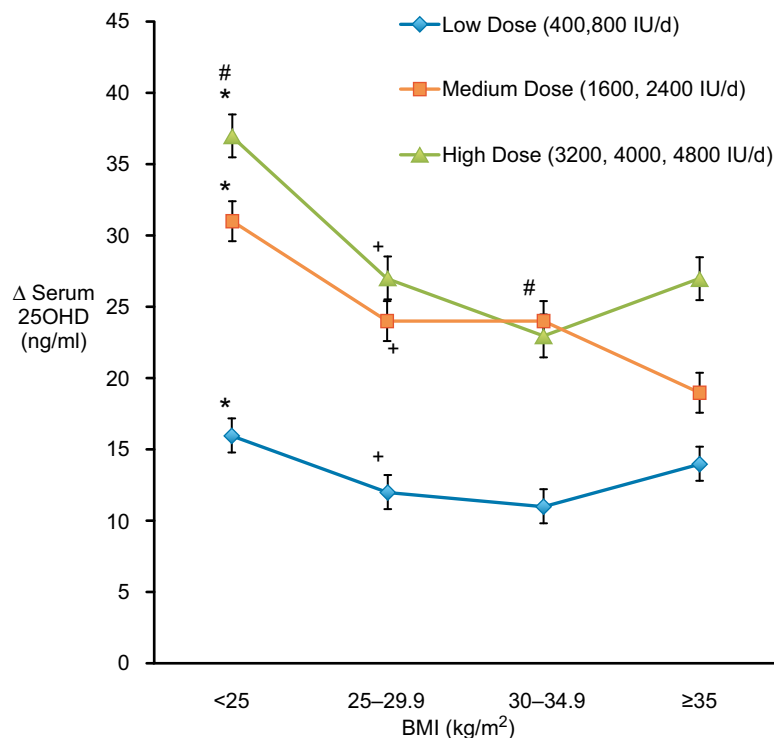
It is of interest that even in a trial just published on the effects of supplementation on obesity in older adults (>65 years old and with concomitant calcium supplementation) who had baseline serum 25(OH)Ds of 10–30 ng/mL and where the average doses given were ~3750 IU/day, achieved serum 25(OH)D concentrations were not reported even though other work has shown it can take much higher doses of vitamin D to achieve repletion in obesity than in the nonobese [69] (see the next section).

## 7. Obesity and the efficacy of oral vitamin D supplementation

Responses of serum 25(OH)D concentration to vitamin D supplementation with specific doses are three to four times higher in some trials than others [70]. Differences in adiposity/body weight account for some of this between-study variation in response since obese individuals require larger doses of vitamin D than lean subjects to achieve equivalent 25(OH)D concentrations. Gallagher and colleagues, for example, tested seven doses of vitamin D, increasing from 400 to 4800 IU/day, and compared final serum 25(OH)D values after supplementation in normal-weight, overweight, and obese women [71]. That trial showed that the response

to supplementation across all dosages was notably greater in normal weight (BMI <25) compared with obese women [71]. However, despite consistently lower responses in the obese compared with normal-weight participants, there was no evidence for any dose–response effects in the variation in 25(OH)D responses with increasing severity of overweight or obesity (Fig. 75.2). In addition, Heaney et al. [72] showed that fat mass was not superior to weight in predicting 25(OH)D responses to supplementation, which suggests that both adipose tissue and skeletal muscle provide significant storage for 25(OH)D (see Chapter 29).

Based on comprehensive systematic review and metaanalyses, Zitterman et al. combined data from 94 independent trials and demonstrated a nonlinear logarithmic association between daily vitamin D dose (per kg total body weight) and the resultant increase in serum 25(OH)D concentrations [73]. In addition to body weight, they identified age as a key predictor for the response to supplementation; in their evidence synthesis, the trials in older people typically reported greater increments in serum 25(OH)D compared with the trials in younger people. This may be due to higher baseline 25(OH)D concentrations in younger people, although the authors suggested that these differences may, in part, reflect age-related changes in calcium and vitamin D physiology [73]. One of the stated aims of that report by Zitterman and colleagues was to develop a formula to establish vitamin D dosages required to



**FIGURE 75.2** The effect of body mass index (BMI) on response to vitamin D supplementation at different dosages. *Reproduced from Ref. [71].*

reach certain target 25(OH)D concentrations in people of different sizes. Fig. 75.2 [73] shows the predictions for the incremental vitamin D dosages required by vitamin D-deficient individuals weighing 50, 75, and 100 kg to reach an adequacy status defined by endpoint 25(OH)D concentrations of either  $>50\text{ nmol/L}$  or  $>75\text{ nmol/L}$ . There are other vitamin D dose prediction formulae giving slightly different estimations. Drincic and colleagues [74] used data from a group of clinically obese (BMI 30–58), but largely vitamin D-replete individuals with an average baseline 25(OH)D value of  $58\text{ nmol/L}$ , and developed a predictive equation based on studies using relatively high vitamin D intakes (at 1000 IU/d, 5000 IU/d, and 10,000 IU/d). According to their formula, the supplemental daily vitamin D<sub>3</sub> doses to give (in IU/d) can be estimated as  $[(\text{weight (kg)} \times \text{desired change in 25(OH)D (in nanograms per milliliter)} \times 2.5)] - 10$  [74]; based on this estimation, the predicted dosages required are notably higher. For example, for a  $25\text{-nmol/L}$  increase in serum 25(OH)D in an individual weighing 75 kg, a dosage of 1865 IU/day is suggested.

Ekwaru and colleagues [75] used information from a large community sample with 22,214 cross-sectional assessments of serum 25(OH)D to estimate the dose-response relationship between oral vitamin D supplementation and achieved 25(OH)D concentrations; the sample was derived from a database including volunteers taking part in a preventative health program, with an emphasis on providing advice in relation to vitamin D supplementation. In this group, participants were self-supplementing with up to 50,000 IU of vitamin D per day, and the baseline 25(OH)D concentrations were also relatively high ( $90.5\text{ nmol/L}$ ). The estimated response to supplementation showed an exponential pattern, with noticeably greater increases in serum 25(OH)D seen in response to smaller as compared with higher dosages. However, in this group, where vitamin D intakes and 25(OH)D concentrations were higher than in most other study populations, 25(OH)D concentrations were again found to be lowest in those individuals who were obese or overweight, as were the increases associated with supplementation. Based on these data, the authors recommended that supplemental vitamin D intakes for obese individuals should be two to three times higher than for those of normal weight, and 1.5 times higher for overweight individuals [75]. Another study in obese individuals (BMI  $>30$ ), which had treated all participants to have serum concentrations at  $\sim 34.0\text{ ng/mL}$  ( $84\text{ nmol/L}$ ) before dividing them into different treatment groups, found that intakes of 2000 IU/day were not enough to retain concentrations at this level over 6 months, while in the group receiving 125 IU/kg/day, the levels remained stable [76].

A retrospective chart review of obese male and female adolescents with vitamin D insufficiency (25(OH)D  $20\text{--}30\text{ ng/mL}$ ) suggested that treatment with 800 IU/day was not enough to increase and retain concentrations  $>30\text{ ng/mL}$  [77]. This study also reviewed adolescents with vitamin D deficiency ( $<20\text{ ng/mL}$ ) who had received a weekly dose of 50,000 IU over 6–8 weeks, which was sufficient to raise average concentrations from  $16\text{ ng/mL}$  to  $23\text{ ng/mL}$ , with 28% reaching  $>30\text{ ng/mL}$ . This fits with the limited data available from studies in children and adolescents, which suggest poor 25(OH)D responses to supplementation in obesity [78]. In those aged 12–18 years, for example, rises in serum 25(OH)D with vitamin D at 2000 IU/day were  $\sim 2$  times greater in normal weight than in obese subjects [78], suggesting that supplemental vitamin D requirements for obese as compared with normal-weight children should be doubled, much as is suggested for adults. Small, but significant, increases in total serum calcium concentrations were seen, without any adverse effects, in the normal weight but not the obese children taking 2000 IU/day, a finding suggesting that body weight should be allowed for when supplementing children with vitamin D in amounts above the currently recommended daily intakes for children, as is standard practice when prescribing medication for children.

## 8. Brown adipose tissue, obesity, and vitamin D

Brown adipose tissue is prominent in hibernating animals, generating heat from fatty acid oxidation and increased uncoupling protein-1 (UCP-1) activity. While brown adipose tissue was long thought to be active only in early life in humans, it is now known that “beige” adipocytes remain in place in various sites and develop brown adipocyte functions under cold or other types of stress [79]. Both brown and white adipose tissue generate proinflammatory cytokines [80], which must increase obesity-related health risks. Brown adipose tissue deposits form during human embryogenesis from precursor cells that also form myocytes (induced by the “myogenic factor 5 promoter”). This effect increases with high-physiological levels of  $1,25(\text{OH})_2\text{D}$  [81] and likely contributes to the recovery of muscle strength with timely correction of vitamin D deficiency, since skeletal muscle atrophy with adipocyte infiltration is a feature of chronic vitamin D deficiency. It is also likely to contribute to the improved myocardial function seen with correction of deficiency in heart failure [82,83].

Adipose tissue UCP-1 expression is inhibited by VDR binding to negative-response elements in the UCP-1

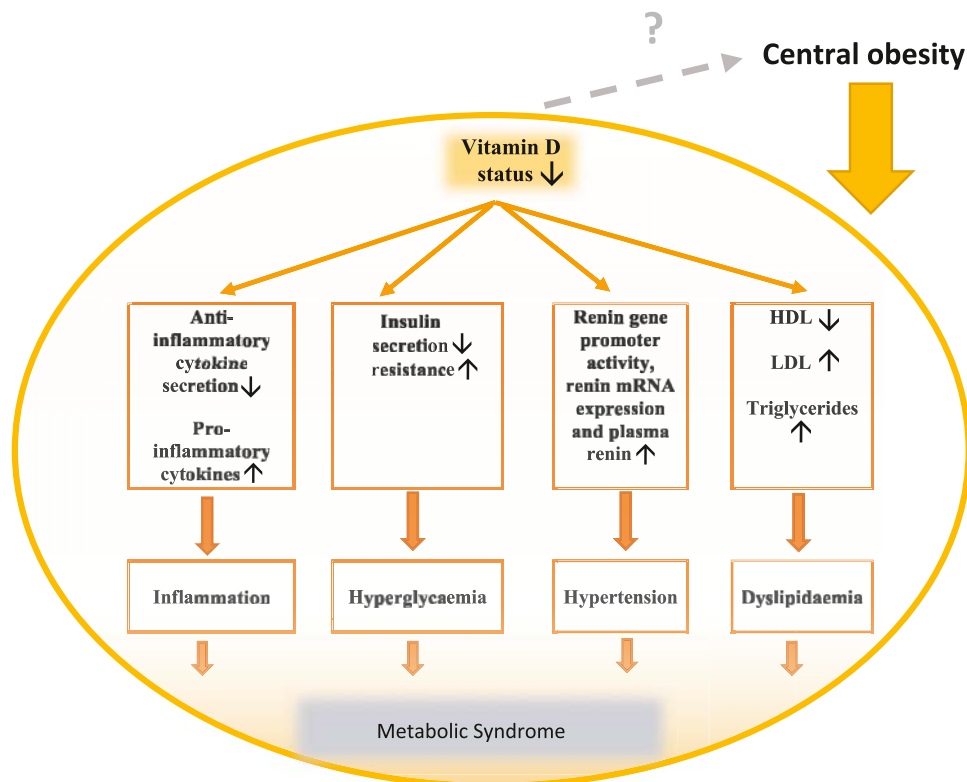
gene promoter region, and  $1,25(\text{OH})_2\text{D}$  suppresses brown adipose tissue differentiation, dose-wise [80,81]. Thus, defects in the VDR and lack of vitamin D can increase the “browning” of human white adipose tissue. Specific adipose tissue VDR knockout (VDR-KO) has, therefore, been suggested as a potential measure for increasing energy expenditure to assist in weight loss [79,82,84,85].

Sizable brown adipose tissue deposits in newborn humans provide valuable thermogenesis but become minimal in adults and show seasonal variation with increases in winter, likely because VDR activation by activated vitamin D suppresses brown fat activity and serum  $25(\text{OH})\text{D}$  normally increases in the summer [86,87]. Increasing brown adipose tissue formation, which could increase energy expenditure has, as mentioned before, been considered as a potential treatment for obesity. However, while brown adipose tissue is scattered throughout white adipose tissue in ~10% of normal children under 10 years old, this is not usually seen in later childhood or in adult obesity, though beige fat deposits persist in adults [79,88]. Hence, ways to promote browning of adipose tissue generally might need to begin in early childhood, while targeted activation of beige fat could increase energy consumption at any age.

## 9. Obesity, vitamin D, and metabolic syndrome

In brief, cross-sectional and prospective associations between vitamin D nutrition and metabolic syndrome (MetS) are well known, and low serum  $25(\text{OH})\text{D}$ s are also related dose-wise to increased risks of MetS, T2DM, and CVD prospectively [89–93] (see Chapter 26). Vitamin D nutrition has mechanistic effects protective against MetS disorders through direct and indirect effects, many contributing directly to the disorders associated with obesity (Fig. 75.3).

Adipose-specific VDR-KO in mice causes increased growth rates and increases visceral adiposity, mainly in females, with its associated adverse effects on health [90,91,94,95]. There is also some evidence suggesting greater reductions in serum  $25(\text{OH})\text{D}$  with visceral than subcutaneous adiposity, but with reductions in insulin resistance and inflammation in obesity with higher vitamin D status [95–98]. Since vitamin D deficiency appears to increase the risks of MetS abnormalities, T2DM and CVD, adequate vitamin D status should reduce obesity-related health risks even without weight loss. Clinical studies aiming to establish causality for the effect of increasing  $25(\text{OH})\text{D}$  concentrations and MetS abnormalities, CVD and T2DM have typically provided little evidence for an effect. This may be due to a



**FIGURE 75.3** Conceptual framework for the proposed joint effects of central obesity and low vitamin D status on the aggravation of metabolic abnormalities and disease risk. CVD, cardiovascular disease; NAFLD, nonalcoholic fatty disease of the liver; T2DM, Type 2 Diabetes mellitus.



combination of increases in 25(OH)D being diluted into the enlarged fat mass, combined with reduced synthesis of 25(OH)D in obesity that is due to suppression of the hepatic 25-hydroxylase enzyme [3,4]. While it has been suggested that the association between vitamin D and cardiovascular risk is merely due to confounding [99], at the physiological and biochemical level, there is compelling mechanistic evidence showing that 1,25(OH)<sub>2</sub>D inhibits many of the adverse effects of an enlarged fat mass.

Most clinical trials to date have been of relatively short duration and could well have failed to include individuals with vitamin D deficiency and/or patients in the earliest stages of the metabolic syndrome. Furthermore, many such studies gave calcium supplementation with vitamin D and accepted relatively high, but unknown, amounts of personal vitamin D intakes in both test and control arms during trials. Overall, the known mechanisms of action of vitamin D demonstrate the potential for adequate provision of vitamin D to reduce the severity of the disorders and health risks commonly seen in association with obesity including the other metabolic syndrome disorders. Although marked weight loss can reduce blood pressure, dyslipidemia, and hyperglycemia, often to normal, it is not easy to achieve, nor is it easy to disentangle the effects of inadequate vitamin D repletion from those of obesity in practice [100].

Prospectively, there are lower overall risks of incident metabolic syndrome and of T2DM after 10 years of follow-up in those with higher baseline vitamin D status compared with those with lower baseline status (e.g., 93). Evidence continues to accumulate to show that better provision of vitamin D reduces abnormal insulin resistance, a recognized risk marker of increased risks of both T2DM and CVD, as well as promoting glucose homeostasis. Problems with randomized control trial (RCT) design continue to reduce the chance of their providing definitive findings [100]. One would, however, predict that optimal vitamin D status should be protective against T2DM as recently reported among those with prediabetes [45] and against CVD from the reported mechanistic effects.

In addition, genetic evidence from MR studies support possible benefits from lifelong increases in serum 25(OH)D with respect to lower risks of T2DM, CVD, and MetS [68,101,102]. Recent findings using a novel nonlinear design, which allows researchers to examine the effects of increasing 25(OH)D concentrations in the context of vitamin D deficiency, suggested notable benefits with respect to lower blood pressure and for CVD risk with increases in serum 25(OH)D up to ~50 nmol/L, with little changes with further increases in 25(OH)D concentrations above ~50 nmol/L [101]. Since all nutrient effects, including health effects of

vitamin D, demonstrate threshold behavior, nonlinear methodology gets around the usual problems in conventional MR analyses that arise from the often false assumption of linearity of association (i.e., that higher 25(OH)D values have quantitatively similar effects regardless of the vitamin D status of the individual) [103] (see Chapter 61). There have been many reports of associations of vitamin D deficiency with risk of MetS over many decades [105–107] and, in view of the increased mortality associated with obesity and type 2 diabetes, mainly from cardiovascular disease, clarification of the contribution of risk from vitamin D deficiency as compared with that due solely to obesity is badly needed.

## 9.1 Inflammation and hormonal adipokines

The adverse remote effects of obesity result largely from the secretion of adipokines and of proinflammatory cytokines and hormonal factors. The overall immunomodulatory effects of 1,25(OH)<sub>2</sub>D lead to reduction of tissue damage from severe innate immune responses and from prolonged adaptive (acquired) immune responses. 1,25(OH)<sub>2</sub>D suppresses proinflammatory factor secretion and concomitantly promotes the secretion of antiinflammatory cytokines [97,108–112]. Particularly relevant to obesity is the reduction by 1,25(OH)<sub>2</sub>D of proinflammatory cytokine secretion by the large accumulations of macrophages that develop in the enlarged visceral fat deposits and that are known to induce remote inflammatory effects in other tissue including the vasculature and nervous system [111]. Such benefits are mediated, in part by suppression of the NF- $\kappa$ B pathway, leading to reduction in the secretion of proinflammatory cytokines such as IL-6 [111,112]; indeed, many genes are well known to be modulated through VDREs on their promoter regions [113]. Macrophage-derived cytokines upregulate the VDR as well as inducing inflammation, providing the opportunity for feedback regulation that would tend to reduce obesity-induced inflammatory tissue damage in vitamin D adequacy; however, information on whether supplementation does provide clinical benefits in obesity, and what vitamin D intakes and serum 25(OH)D values are necessary for optimal health benefit in obesity remains unclear [114].

Since inflammation is seen in adipose tissue and has major remote effects increasing CVD and T2DM risks, the analysis of UK biobank data using the nonlinear MR methodology that has revealed reductions in CVD risks with genetically determined increases in serum 25(OH)D among those with vitamin D inadequacy at baseline is of current interest [101]. A recent study has provided further direct evidence for a causal link

between vitamin D deficiency and increased inflammatory atherosclerosis [115]. Furthermore, bidirectional MR analysis has recently shown sharp decreases in serum CRP levels with increases in genetically determined 25(OH)D levels that are inversely associated with serum 25(OH)D at values < 25 nmol/L and with a similar, but less marked, association in subjects with insufficiency (25(OH)D values between 25 and 50 nmol/L), but no association at values > 50 nmol/L [116]. This suggests that correction of vitamin D deficiency can help reduce systemic low-grade inflammation, potentially mitigating obesity-related health risks including cardiovascular disease [116].

Adipokines produced by adipose tissue have variable effects, leptin and adiponectin improving insulin sensitivity while others increase the risk of glucose intolerance and of the metabolic syndrome in general [117]. Adipose tissue macrophages also secrete cytokines such as TNF- $\alpha$  and IL-6, which contribute to worsening adiposity-associated inflammation remotely, as in atherosclerosis, as discussed before. Metaanalysis of RCTs has not suggested an overall effect of vitamin D supplementation on serum leptin concentrations, but correlations are common between changes in leptin and in 25(OH)D concentrations. Leptin, a circadian appetite suppressant secreted by adipose tissue, reduces food intake and body weight but is not used to control obesity, though potential ways to exploit its effects remain of interest [118].

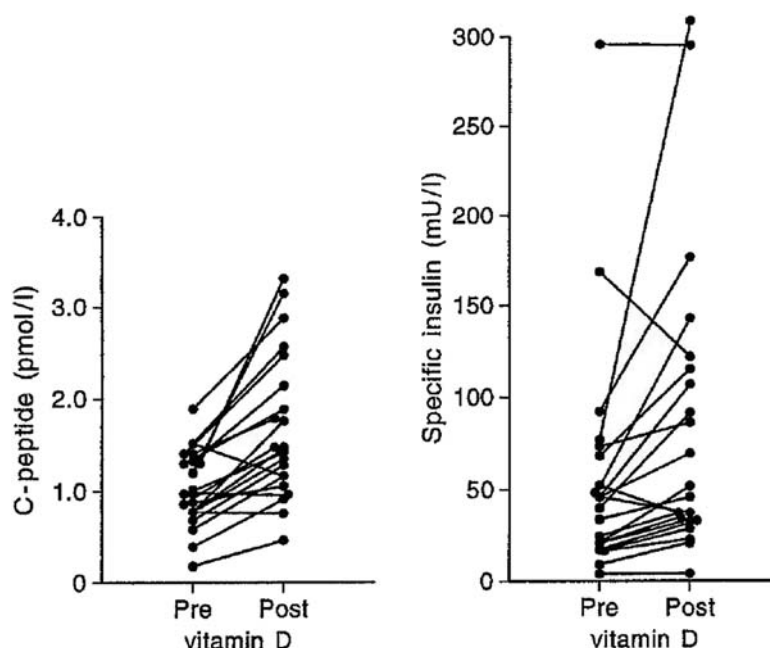
Cord blood leptin is raised in gestational diabetes but inversely associated with cord blood adiponectin, which is associated with neonatal adiposity [118,119]. Similarly in adults, circulating concentrations of adiponectin also relate inversely to obesity and directly to serum 25(OH)D with genetic evidence for a causal association; 1,25(OH)<sub>2</sub>D stimulation of adiponectin secretion [120,121] should reduce insulin resistance and MetS and T2DM risks since review of the literature shows that adiponectin increases tissue glucose uptake and fatty acid oxidation, thereby increasing insulin sensitivity in liver and muscle [120]. Despite this, RCTs report no increases in plasma total adiponectin concentrations with vitamin D supplementation [121] though many were small (only one included >100 participants), of short duration, and with limited data on adipokine profiles. One exception was the auxiliary study to the Vitamin D Assessment (ViDA) study, giving 2000 IU/day of vitamin D (vs. placebo) during lifestyle-based weight loss over 1 year [122], which did not find improvements in the eight inflammatory biomarkers studied (or in their composite score). A secondary stratified analysis, however, looking solely at those who lost weight, suggested that those taking vitamin D had greater reductions in circulating IL-6 (but not of other cytokines) versus those on placebo [122].

From intrauterine life onward, insulin-like growth factor (IGF-1) and its binding proteins (IGF-1BPs) are secreted by adipose tissue, including preadipocytes [123], and have some effects on adipose tissue development and also reduce insulin resistance (IR) through their effects on liver and muscle. Serum IGF-1 concentration associates inversely with MetS risk in the vitamin D-replete subjects among the ~6800 people being followed in the 1958 BCE, aged 45 years [38]. This is of interest since 1,25(OH)<sub>2</sub>D upregulates IGF-1 secretion, and IGF-1 upregulates vitamin D activation, a feedback cycle likely to enhance the efficacy of both IGF-1 and vitamin D [124].

## 9.2 Insulin resistance, insulin secretion, and glycemia

Increased insulin resistance (IR) develops in obesity where increased adiposity of both liver and muscle is usual. Vitamin D has been known for ~40 years to be necessary for normal insulin responses to glucose [125–127]. Insulin release from storage granules in healthy islet beta cells results from rapid nongenomic effects increasing intracellular calcium content (phase 1) following VDR activation in islet beta cell wall caveolae by 1,25(OH)<sub>2</sub>D. This effect is followed by slower genomic effects increasing insulin synthesis that follow 1,25(OH)<sub>2</sub>D activation of nuclear VDRs (phase 2) [125,126], which depends on dietary vitamin D in isolated rat islets experimentally [125–127]. Long-term increases in IR eventually lead to the well-known beta-cell failure, beta cell death, and islet fibrosis that eventually cause overt T2DM.

Protective effects of vitamin D against T2DM should, therefore, be the greatest before irreversible pancreatic islet damage develops, a postulate supported by the dose-wise reductions in MetS and in T2DM risks seen prospectively with higher baseline vitamin D status after 10 years [91,92]. Further support comes from the finding that glucose-stimulated secretion of insulin (GSSI) with vitamin D supplementation was greater, as a dose-effect, in T2DM subjects with shorter durations of T2DM [128]. These various data suggest that early vitamin D repletion should maximize risk reduction for T2DM. The need to establish vitamin D repletion early for reducing T2DM risks was also suggested by the finding of larger increases in 30 min GSSI on oral glucose tolerance testing in those with the higher, and thus the most normal, GSSI responses presupplementation [129] (Fig. 75.4). The total reversal of new-onset glucose intolerance in renal failure by 1,25(OH)<sub>2</sub>D treatment also supports the concept that the earlier the correction of vitamin D deficiency, the more likely the reduction in the risks of hyperglycaemia and type 2



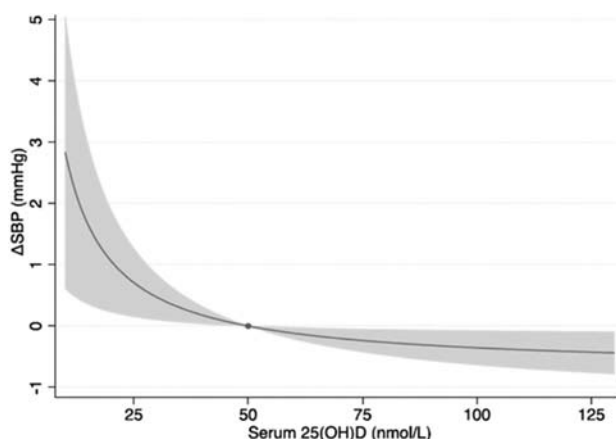
**FIGURE 75.4** Individual changes in C-peptide and specific insulin (30 min OGTT concentrations) before and after vitamin D administration in 22 individuals "at-risk" of diabetes (spot blood glucose  $>6$  mmol/L less than 2 h after food or  $>4.6$  mmol/L more than 2 h after food). Reproduced from Ref. [129].

diabetes [130]. Additionally,  $1,25(\text{OH})_2\text{D}$  is protective against UCP-2 overactivity-induced apoptosis of pancreatic islet cells, and against hyperglycemia-induced renin–angiotensin–system overactivity, functions protective for islet beta cells as hyperglycemia develops [131].

RCTs with adequate vitamin D supplementation can reduce abnormally raised insulin resistance, and T2DM risk in those with prediabetes (e.g., [45]) although these benefits of better D status have not yet been confirmed in obesity and require further study. Vitamin D reduces hepatic lipid formation and glucose output under insulin-resistant conditions experimentally [132], but RCTs do not always reproduce these effects, probably due to inadequate numbers of deficient subjects recruited and inadequate supplementation to correct those with deficiency [133]. Informatively, a study in 81 vitamin D–deficient insulin resistant, but normoglycemic, South Asian women in New Zealand given 4000 IU/day for at least 6 months showed significant improvements in insulin sensitivity and falls in IR (with no changes in C-peptide or glycemia), which only appeared when serum  $25(\text{OH})\text{D}$  concentrations reached 80–119 nmol/L [105], a finding suggestive of a threshold effect at  $\sim 80$  nmol/L. This is in line with cross-sectional data on 239 obese black and white women without diabetes, whose indices for glycemia and IR were minimal with serum  $25(\text{OH})\text{D}$  values  $\geq 65$  nmol/L [134].

### 9.3 Vitamin D and hypertension

Since hypertension aggravates cardiovascular disease (see Chapter 26) and is itself aggravated by obesity, and since it contributes to the ill health associated with obesity and the metabolic syndrome, the question as to whether vitamin D repletion can reduce the blood pressure in hypertensive patients is of importance. As discussed earlier, renin secretion is suppressed by  $1,25(\text{OH})_2\text{D}$  and knockout of the vitamin D receptor increases systolic blood pressure [135,136]. Despite these mechanistic effects, many trials of vitamin D supplementation in hypertension have failed to reduce the blood pressure [137]. However, typically, such trials have been conducted in individuals who are relatively vitamin D replete, while MR evidence suggests that the association may be causal and also suggests that benefits may be largely restricted to the correction of deficiency [101,138]. In Fig. 75.5, we show how increases in  $25(\text{OH})\text{D}$  concentrations associate with lowering of systolic blood pressure in participants who have concentrations below 25 nmol/L (as compared with being at or above 50 nmol/L), and how this association plateaus above 50 nmol/L [101]. This finding is in line with an earlier linear MR study that has already provided genetic evidence for a causal effect of higher  $25(\text{OH})\text{D}$  with both a lower blood pressure and with reductions in the risk of hypertension [138]. Thus, the increased CVD risk due to concomitant hypertension in obesity should be reduced if vitamin D deficiency can be



**FIGURE 75.5** Genetic association of serum 25-hydroxyvitamin D with systolic blood pressure. The dot represents the reference point of serum 25-hydroxyvitamin D of 50 nmol/L. The shaded areas represent the 95% confidence intervals. Adjustment includes age, sex, assessment centre, birth location, single nucleotide polymorphism array, Top 40 genetic principal components, and nuisance factors which could affect serum 25-hydroxyvitamin D measurements, including month in which blood sample was taken, fasting time before blood sample was taken, and sample aliquots for measurement. *Reproduced from Ref. [101].*

avoided over time. Interestingly, reductions in blood pressure and in CVD risks have now been reported by secondary MR analysis in subjects with both diabetes and hypertension [139].

#### 9.4 Dyslipidemia and nonalcoholic fatty disease of the liver

Fatty acids formed specifically in the white adipose tissue are increased in length by fatty acid elongases, and one of these (Elovl3) is downregulated by 1,25(OH)<sub>2</sub>D through negative-response elements in the promoter region of its gene [140]. Inverse associations of vitamin D status with the severity of nonalcoholic fatty disease of the liver (NAFLD) are widely reported, and with its serious sequelae of cirrhosis and primary liver cancer [141,142], though not in a study of obese children under 18 years, apart from the evidence of hepatic inflammation [143]. In high-fat:high-glucose diet-fed rodents and in hepatocytes, *in vitro*, 1,25(OH)<sub>2</sub>D has been shown to promote hepatic lipid homeostasis by increasing free fatty acid metabolism, thereby reducing hepatic TAG formation and hepatic steatosis, and to reduce hepatic glucose output [132]. These effects could help to explain why increased severity of steatosis and other features of NAFLD are reported with lower serum 25(OH)D concentrations [141–147]. In a recent systematic review and metaanalysis, an RCT was mentioned in 60 patients with NAFLD, given 50,000 IU vitamin D<sub>3</sub> weekly for 10 weeks versus placebo; mean serum

25(OH)D rose significantly on vitamin D (to 70 nmol/L), with a concomitant fall in IR (estimated as homeostatic model assessment HOMA-IR) [148]. In the controls, there was also a small rise in 25(OH)D, and though there was no change in estimated insulin secretory response to glucose (HOMA-beta) in controls, there was a small fall in fasting glucose, suggesting that this is a consistent effect. However, the inverse dose-dependent relationship between vitamin D status and NAFLD has also been found in vitamin D repletion suggesting some degree of reverse causality in this association as would be expected with hepatocellular disease since the liver is a major site of vitamin D 25-hydroxylation. These data are suggestive of potential benefits of giving vitamin D therapeutically in NAFLD. The aforementioned review and metaanalysis of data from 16 trials of vitamin D supplementation in NAFLD has shown that supplementation increased serum HDL cholesterol, reduced body weight and BMI, waist circumference, serum alanine-transferase, HOMA-IR estimates of insulin resistance, and fasting blood glucose but without changes in other circulating lipids, in other liver enzymes or in serum adiponectin [148]. This data led the authors to suggest maintaining vitamin D repletion as an effective strategy for the management of NAFLD [148]. Whatever effect repletion may or may not have on NAFLD itself, this data suggests that vitamin D repletion should reduce the general health risks associated with NAFLD through adequate provision of 25(OH)D to the target tissues, as can be expected in any disorder that reduces serum 25(OH)D concentrations.

Lipid profiles have been reported to be variously affected by vitamin D supplementation over many years. Review and metaanalysis have shown beneficial effects of vitamin D, often in combination with calcium, with consistent improvements in serum triacylglycerides but only minimal improvements, if any, in HDL and LDL cholesterol in most studies [149,150]. Such small effects might reflect lack of benefit or inadequate trial design since a more recent review and metaanalysis suggest more marked benefits. Definitive studies are needed in this area.

#### 10. Importance of early-life vitamin D status for later obesity and subsequent risks of metabolic syndrome, type 2 diabetes mellitus, and cardiovascular disease

Maternal vitamin D metabolism changes during pregnancy [151] (see Chapters 32–34). Although circulating 25(OH)D crosses the placenta poorly [152], there is a marked increase in the activation of 25(OH)D to 1,25(OH)<sub>2</sub>D during early pregnancy within pregnancy



-specific tissues, i.e., the decidua and placenta, and maternal blood  $1,25(\text{OH})_2\text{D}$  concentrations double by the third trimester [153]. At the same time, normal homeostatic downregulation of serum  $1,25(\text{OH})_2\text{D}$  is lost because of epigenetic inactivation of the catabolic enzyme CYP24A1 [154] in conceptual tissue. These changes help to ensure that adequate amounts of  $1,25(\text{OH})_2\text{D}$  are available in the maternal circulation to supply the needs of the developing fetus.

The epigenetic effects of poor maternal nutrition are increasingly well recognized. Maternal vitamin D inadequacy leads to variations in methylation of the promoter region of the calcium transporter gene [155,156], with likely effects on offspring due to changes in the mechanisms relevant to bone health and growth. Furthermore, vitamin D has many other epigenetic effects; thus, early-life vitamin D status can contribute to the determination of later nonskeletal health risks, in ways that cannot be identified by RCTs of supplemental vitamin D later in life. For example, epigenetic effects of experimental maternal vitamin D deficiency persist into adulthood, with long-term effects on the liver and pancreas [58,156,157]. Similarly, children born to vitamin D-deficient mothers were more obese as neonates and aged 1 year as compared with children born to replete women [155–157]. It is generally accepted that many disease risks are transmitted across generations through epigenetic mechanisms. For example, poor intrauterine nutrition, maternal obesity, maternal T2DM, and gestational diabetes (whose prevalence is increased by maternal vitamin D deficiency) are all well-recognized risk factors for increased T2DM risks in human offspring later in life [158–160].

Nutritional “imbalances,” and factors such as paternal smoking and betel chewing, contribute to offspring risks of metabolic syndrome and T2DM, and vitamin D status could affect these risks. Evidence for transgenerational transmission of such effects has been obtained, for example, from human data and from mouse offspring of vitamin D-deficient dams, which showed increased weight, hepatic fatty acid synthase activity, IR, increased pancreatic islet size, and hepatic steatosis [59,161–163].

There are many putative explanations of how early-life vitamin D deficiency could increase offspring obesity risk, including increases in adipogenesis [162] associated with the inhibition of adipocyte maturation into myocytes, aggravated by early obesity-induced increases in adipocyte cell numbers and size that appear to be irreversible, and the induction of permanent changes to hypothalamic appetite regulation [163–165]. Specifically,  $1,25(\text{OH})_2\text{D}$  upregulates the VDR in developing adipocytes, and VDR activation inhibits adipocyte maturation [166], likely favoring their differentiation into myocytes. Seasonal changes in

temperature and solar UVB radiation could also influence these associations; the “winter hypothesis,” suggesting that low winter UVB radiation reduces skin synthesis of vitamin D, signals the need for energy storage, which increases body weight, optimizes heat conservation, and increases tolerance of winter food shortages [167]. However, studies on associations between maternal vitamin D status and offspring fat mass produce inconsistent findings; for example, in two studies in the United Kingdom, one found low-maternal vitamin D status to be associated with greater offspring fat mass at 4 and 6 years of age, while another study from the same geographical area suggested lower offspring fat mass aged 9 years [168,169]. In a societal experiment on the effects of food fortification with vitamin D in Denmark, no differences in body size were seen at 7 years of age between children born before or after implementation of the program [170]. Regardless of the apparent absence of effects of short-term supplemental increases in vitamin D intakes on obesity risks, maternal and early-life vitamin D status could have important influences on later life risks of metabolic abnormalities associated with obesity, including inflammation, dyslipidemia, hypertension, hyperglycemia, overt type 2 diabetes, and cardiovascular disease. Long-term vitamin D repletion may, therefore, provide some protection from the health risks of obesity over the life span. This concept is further supported by the many mechanisms identified as underlying the associations of obesity and the resultant vitamin D deficiency with the health risks, as already discussed, as well as contributing to later ill-health through in utero “programming” of offspring ill health by maternal vitamin D deficiency.

Overall, the various associations between vitamin D insufficiency and obesity-related metabolic abnormalities are very similar in children, adolescents, and adults. Since the development of obesity, its related metabolic abnormalities, and atherosclerotic vascular disease, all begin in childhood and are progressive throughout life, anything that worsens these risks over the life span will increase the risks of these disorders becoming clinically obvious in adults [171,172]. Follow-up of offspring in cohorts where maternal vitamin D status was assessed, in RCTs where maternal vitamin D supplementation has been given, and of populations where vitamin D deficiency has been virtually abolished, as in Finland [173], could eventually clarify the contribution of early-life vitamin D status to later health risks. This is especially important for offspring outcomes that may not be changed by later supplementation or where structural damage has already begun before disease is apparent, as in both T2DM and CVD. Overall, it is plausible that long-term repletion with vitamin D from early in life may have a key role in reducing later health risks.

Childhood obesity is a key determinant of later Mets risks, and the increased health risks in obesity must be aggravated, since the reduced serum 25(OH)D that obesity induces must worsen the provision of 25(OH)D substrate to the tissues for the formation of 1,25(OH)<sub>2</sub>D.

## 11. Obesity, vitamin D, and cancer

Obesity is a well-recognized risk factor for various types of cancer, and it is possible that the reduced circulating 25(OH)D of obesity contributes to these risks [174,175]. How far the increased cancer risks in obesity reflect the effects of the reduced circulating 25(OH)D seen in obesity as discussed before is an important question since lowered circulating 25(OH)D reduces the supply of 25(OH)D to target tissues as a substrate for 1,25(OH)<sub>2</sub>D synthesis and promoting auto- and paracrine functions of vitamin D. Ensuring vitamin D adequacy (by food fortification + targeted supplementation [173]) would be simpler and more cost-effective approach than abolishing the obesity, though overweight/obese people would need higher supplemental intakes than others [75].

## 12. Obesity, COVID-19, and vitamin D

Being overweight or obese is a recognized risk factor for developing severe COVID-19 illness and was associated with increased COVID-19 mortality rates in the recent pandemic. Excess weight is also thought to be a factor leading to the increased severity of this illness in British ethnic minority groups where overweight is a common problem as are deprivation, type 2 diabetes, and vitamin D deficiency [176–178]. COVID-19 often leads to acute pulmonary inflammation, with or without secondary infection, and can develop into the acute respiratory distress syndrome (ARDS), with excessive immune responses (cytokine storm) and high death rates. Such problems increase with inadequate secretion of defensins and cathelicidin, both of which are stimulated by 1,25(OH)<sub>2</sub>D. ARDS worsens with failure to suppress the secretion of proinflammatory cytokines (e.g., IL-6) in combination with reduced production of antiinflammatory cytokines (e.g., IL-10), while 1,25(OH)<sub>2</sub>D has beneficial effects on both those problems [178,179] (see Chapter 99).

Circulating MMPs, mainly MMP2/9, increase in severe, and remain raised in prolonged, COVID-19 illness [180,181]. Targeted MMP2/9 reduction is suggested for treatment of COVID-19 and could potentially also help with long COVID. Vitamin D supplementation reduces abnormally raised circulating MMP2/9 [182]. In ARDS,

pulmonary ACE-2R secretion falls, leading to worsening lung damage. However, vitamin D promotes ACE-2R secretion, specifically in the lungs where ACE-2R has protective effects against ARDS [179]. Overall, therefore, being vitamin D adequate before infection should reduce the risk of illness as has been reported from a large US study where better prepandemic D status was associated dose-wise with reducing infection rates by up to 50% once 25(OH)D values reached ~ 50 ng/mL [~125 nmol/L] [183]. Similar evidence for protection was found in a study from Israel with additional data showing a ×14-fold reduction in the risk of COVID-19 death with previous vitamin D adequacy [184].

In a UK biobank data analysis, obesity, minority ethnicity, and social deprivation were predictors of COVID-19, but adjustment for obesity and ethnicity abolished vitamin D status as a predictor, though those adjustments may be unhelpful since these factors are major determinants of vitamin D deficiency [185–187]. Additionally, though rapidly raising serum 25(OH)D levels have been tested for reduction in COVID-19 risks, benefit has not been reported, even with large doses of vitamin D<sub>3</sub> [188], while benefits have been reported in patients given calcifediol [25(OH)D] in several small studies, including an observational cohort study that suggests a large reduction in mortality [188,189]. This apparent paradox is explained, and is predictable, since rises in serum 25(OH)D with vitamin D treatment are slow (days/week), while serum 25(OH)D rises rapidly with calcifediol treatment (within hours). Furthermore, large bolus doses of D<sub>3</sub> activate autoregulatory mechanisms normally acting to prevent vitamin D toxicity that specifically reduce vitamin D activation [190,191]; those effects persist for at least 3 months, and this form of treatment is known to allow rickets to develop in deficient children (see Chapters 62 and 63). There are, however, no data available from large and adequately designed randomized controlled trials of the use of calcifediol in patients hospitalized with COVID-19, and, until such studies emerge, this form of treatment is unlikely to be accepted for general use.

## 13. Conclusions

A greater volumetric distribution of 25(OH)D appears, at least in part, to be a valid explanation for the lower circulating concentrations of 25(OH)D seen in obesity together with suppression of the hepatic 25-hydroxylation of cholecalciferol that forms 25(OH)D, which also explains the smaller increases in serum 25(OH)D concentrations than expected from studies in normal-weight subjects that are seen with supplementation in obesity. In some, but not all, studies, “free” 25-hydroxyvitamin D concentrations were also lowered in

obesity. Further studies are needed to determine the relative importance of these metabolites in relation to the various health problems associated with obesity in both short and long terms.

Further work is also needed to examine the causality of poor vitamin D status for many more nonskeletal disorders. Similarly, whether the reductions in serum 25(OH)D induced by obesity and diabetes (reverse causality) increase health risks, independently, is an important question since the answer could affect the clinical management of many patients; it could also lead to public health measures for reducing vitamin D inadequacy and hence reduce many health risks prospectively in the many populations where obesity and inadequate vitamin D continue to be common.

Current knowledge on the roles of adequate vitamin D status in the reduction of health risks associated with obesity suggests that maintaining vitamin D adequacy through the life span should significantly reduce the risks of health problems associated with inadequate vitamin D status. However, most RCTs in this area, including those used in metaanalyses, have failed; largely because few subjects with deficiency were recruited into such trials and also because vitamin D dosage was often insufficient to correct deficiency over the duration of the trial. Further high-quality studies that achieve the thresholds reported to be necessary for showing benefits of interest in obesity are still required since the amounts of vitamin D needed to achieve specific target 25(OH)D thresholds in obesity are so much higher in obese than in nonobese subjects. Since the intakes needed to achieve adequate vitamin D status in obesity will also increase with comorbidities such as diabetes and older age, and with the severity of baseline vitamin D deficiency, it will be important to ensure that such trials are correctly designed. Future clinical trials should, for example, define the vitamin D status required for recruitment (a serum 25(OH)D concentration <50 nmol/L) and the vitamin D status that should be achieved (>50 nmol/L), though that target may vary with certain health problems. They should also show that target 25(OH)D concentrations are achieved during trials. While lack of physical activity and unhealthy lifestyle are important risk factors for obesity and for the serious disorders associated with obesity, including the metabolic syndrome, T2DM and CVD, as well as worsening obesity itself, the associated problem of vitamin D deficiency would be much easier to correct (for example, by adequate food fortification plus targeted supplementation) than are lifestyle risk factors such as obesity.

Whatever the value of ensuring vitamin D adequacy may prove to be at the population level, the importance of an effective public health strategy for reducing the risks of metabolic syndrome abnormalities and of CVD

and T2DM is obvious. This strategy should aim to prevent obesity from early in life and include routine normalizing of maternal vitamin D status, since adequate supplies of 1,25(OH)<sub>2</sub>D to the unborn child early in pregnancy reduce later obesity risks. This simple measure would be valuable in supporting other programs aimed at providing all the well-established benefits of weight control.

## 14. Summary points

- Adipose tissue is an important storage site for 25(OH)D, and individuals with obesity are at high risk of low concentrations.
- Weight reduction in the context of obesity is associated with improvements in vitamin D status.
- Obesity affects vitamin D requirement, and while there are different recommendations, intakes that are two to three higher may be required for individuals with obesity compared to normal weight.
- Obesity related vitamin D deficiency may have notable implications for metabolic health, with evidence for increased risks of low-grade inflammation, heart disease, and cancer.

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# The role of vitamin D in type 2 diabetes

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## OBJECTIVES

- Understand the mechanistic pathways that link vitamin D and type 2 diabetes.
- Establish the relationship between low vitamin D status and risk of developing type 2 diabetes and understand limitations of observational studies.
- Evaluate the evidence from clinical trials on the role of vitamin D for the prevention and treatment of type 2 diabetes.
- Synthesize the evidence from the vitamin D and type 2 diabetes prevention trials.
- Recognize the importance of postrandomization biases in trials and the “treat-to-target” concept.
- Discuss the role of adiposity in modulating the effect of vitamin D.
- Examine the safety of vitamin D in people at risk for type 2 diabetes.

## 1. Introduction

Vitamin D has garnered attention for prevention of type 2 diabetes as longitudinal observational studies have shown a consistent association between higher blood 25-hydroxyvitamin D [25(OH)D] levels and lower risk of developing diabetes [1,2]. Potential mechanisms by which vitamin D may affect glucose metabolism include stimulating insulin secretion decreasing systemic inflammation, and improving insulin resistance in muscle and liver [3].

There are three trials that were specifically designed and conducted to test whether vitamin D reduces the rate of progression to diabetes in people with prediabetes [4–6]. This chapter (1) reviews the mechanistic pathways that link vitamin D and type 2 diabetes pathophysiology, (2) describes the consistent association between low vitamin D status and risk of developing type 2 diabetes, (3) synthesize the efficacy-safety evidence from the large vitamin D and type 2 diabetes prevention trials (4) highlights the importance of post-randomization biases in trials and the importance of the “treat-to-target” concept.

## 2. Epidemiology and burden of type 2 diabetes

Diabetes mellitus has become a significant global healthcare problem. According to the United States Centers for Disease Control, about 37.3 million people (about 11.3% of the US population) had diabetes (diagnosed or undiagnosed) in 2019. This total included 37.1 million adults 18 or older, or 14.7% of all US adults. About 8.5 US million adults had diabetes but were not aware that they had it or did not report that they had it [7]. The most common type of diabetes is type 2 diabetes, accounting for over 95% of all diabetes. Type 2 diabetes is often preceded by prediabetes, which is a glycemic state where blood glucose level is higher than normal but not high enough for a diabetes diagnosis. Prediabetes increases a person’s risk of developing type 2 diabetes, heart disease, and stroke. The Centers for Disease Control estimates that 96 million—or more than 1 in 3—US adults aged 18 years or older had prediabetes in 2019. This number includes over 37 million adults aged 45–64 years and over 26 million adults aged 65 years or older. In 2017–20 period, only about 17.4% of people with prediabetes

report that they had ever been notified by a health professional of their prediabetes status; however, this percentage is improved from 6.5% from a decade ago [7]. Diabetes is also an important public health problem worldwide affecting more than 537 million people, and this number is predicted to rise to 643 million by 2030 and 783 million by 2045 [8]. Diabetes is the leading cause of blindness, kidney disease, heart disease, and stroke. In the United States alone, each year up to 25,000 individuals lose their sight, and as many as 28,000 initiate treatments for chronic kidney failure because of diabetes. People with diabetes are four times more likely to develop cardiovascular disease (coronary artery disease, peripheral vascular disease, or stroke) compared with those without diabetes. Beyond its devastating human toll, diabetes is also associated with increasing costs, estimated at more than 300 billion in the United States alone and more than half a trillion dollars worldwide [9].

Although therapies for type 2 diabetes and its complications have improved over the past few decades, the increasing burden of type 2 diabetes highlights the need for innovative approaches for the management and prevention of the disease. Epidemiologic data suggest that 9 out of 10 cases of type 2 diabetes could be attributed to modifiable habits and lifestyle [10]. In clinical trials, lifestyle changes aiming at weight loss are successful at reducing risk of type 2 diabetes [11]. However, long-term weight maintenance in the clinical setting has proved elusive with lifestyle changes alone. Moreover, even after successful weight loss, there is still significant residual risk (~40–50%). Therefore, identification of weight-independent and easily modifiable risk factors is urgently needed to prevent type 2 diabetes and decrease patient and societal burden.

Based on accumulating evidence from animal and human studies (observational studies and clinical trials), as reviewed in this chapter, suboptimal vitamin D status has emerged as a risk factor for type 2 diabetes and vitamin D has been proposed as an intervention when caring for patients at risk for type 2 diabetes.

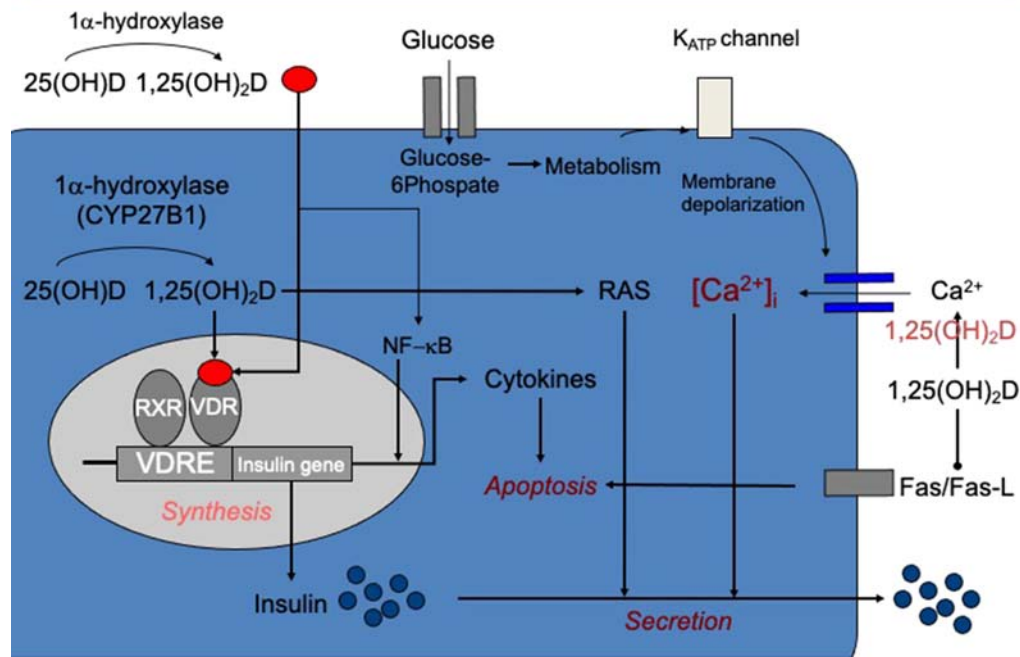
### 3. Biologic plausibility of the link between vitamin D and type 2 diabetes

For glucose intolerance and type 2 diabetes to develop, impaired pancreatic beta-cell function, insulin resistance, and systemic inflammation are often present [12,13]. There is evidence that vitamin D modulates many of these mechanisms, as described next.

#### 3.1 Vitamin D and pancreatic beta-cell function

There are several lines of evidence supporting a beneficial role for vitamin D in pancreatic beta-cell function

(Fig. 76.1) [14–16]. In *in vitro* and *in vivo* studies, vitamin D deficiency impairs glucose-mediated insulin secretion from rat pancreatic beta-cells [17–21], while vitamin D supplementation restores glucose-stimulated insulin synthesis and secretion [17,20–24]. Vitamin D may have a direct effect on beta-cell function mediated by binding of the circulating active form, 1,25(OH)<sub>2</sub>D, to the vitamin D receptor (VDR), which is expressed in pancreatic beta-cells [25,26]. Furthermore, mice lacking a functional VDR show impaired insulin secretory response following a glucose load, attributed to a decrease in insulin synthesis due to a reduction in insulin stored in the beta-cell [27]. In addition, transgenic mice overexpressing VDR in beta-cells were protected against streptozotocin-induced diabetes and had preserved beta-cell mass and a reduction in islet inflammation [28]. Activation of vitamin D also occurs within the pancreatic beta-cell by the 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase enzyme (CYP27B1), which is expressed in pancreatic beta-cells [29]. Such mechanism allows for an important paracrine effect of circulating 25[OH]D. An indirect effect of vitamin D on the pancreatic beta-cell may be mediated via its regulation of extracellular calcium concentration and calcium flux through the beta-cell [30]. Insulin secretion is a calcium-dependent process [31]; therefore, alterations in calcium flux can have an effect on insulin secretion [32–35]. Vitamin D also regulates calbindin, a cytosolic calcium-binding protein found in many tissues including beta-cells [25,36]. Calbindin is a modulator of depolarization-stimulated insulin release via regulation of intracellular calcium [37]. Another potential pathway is through the pancreatic islet renin–angiotensin system (RAS), which appears to play a role in maintaining islet cell mass and insulin secretion [38]. VDR-knockout (KO) mice have increased islet RAS expression compared with wild-type (WT) mice, and 1,25(OH)<sub>2</sub>D prevents and corrects induction of RAS component production under high-glucose conditions with concomitant increases in islet insulin secretion [39]. Finally, vitamin D may promote beta-cell survival by modulating the generation (e.g., through inactivation of nuclear factor- $\kappa$ B [NF- $\kappa$ B]) and effects of cytokines [40,41]. In cross-sectional human studies, an association between blood 25(OH)D level and insulin secretion has been reported in some [42–46] but not all studies [47]. Trials that have reported on the effect of vitamin D supplementation on beta-cell function have been inconclusive [48–51]. The divergent results may be due to differences in populations studied (normal glucose tolerance, prediabetes, or established diabetes), methods of measuring insulin secretion, and beta-cell function and/or variation in the dose and duration of treatment with vitamin D (e.g., intermittent, very high doses of vitamin D are considered nonphysiologic



**FIGURE 76.1 Vitamin D and pancreatic beta cell function.** Vitamin D can promote pancreatic beta cell function in many ways. The active form of vitamin D,  $1,25(\text{OH})_2\text{D}$ , enters the beta cell from the circulation and interacts with the vitamin D receptor-retinoic acid x-receptor complex (VDR-RXR) to enhance the transcriptional activation of the insulin gene and increase the synthesis of insulin. Vitamin D may promote beta-cell survival by modulating the generation (through inactivation of nuclear factor kappa B [NF- $\kappa$ B]) and effects of cytokines. The anti-apoptotic effect of vitamin D may also be mediated by downregulating the Fas-related pathways (Fas/Fas-L). Activation of vitamin D also occurs intracellularly by 25-hydroxyvitamin D- $1\alpha$ -hydroxylase enzyme (CYP27B1), which is expressed in pancreatic beta cells. Vitamin D may also have effects on the expression of cytoskeletal and intracellular trafficking genes along with genes involved in ion transport to influence **insulin exocytosis**. The effects of vitamin D may be mediated indirectly via extracellular calcium ( $\text{Ca}^{2+}$ ), calcium flux through the beta cell and intracellular calcium ( $\text{Ca}^{2+}$ )<sub>i</sub>. Alterations in calcium flux can directly influence insulin secretion, which is a calcium-dependent process. Vitamin D also regulates calbindin, a cytosolic calcium-binding protein found in beta cells, which acts as a modulator of depolarization-stimulated insulin release via regulation of intracellular calcium. Calbindin may also protect against apoptotic cell death via its ability to buffer intracellular calcium. Vitamin D may also correct induction of RAS component production under high-glucose conditions with concomitant increases in islet insulin secretion.

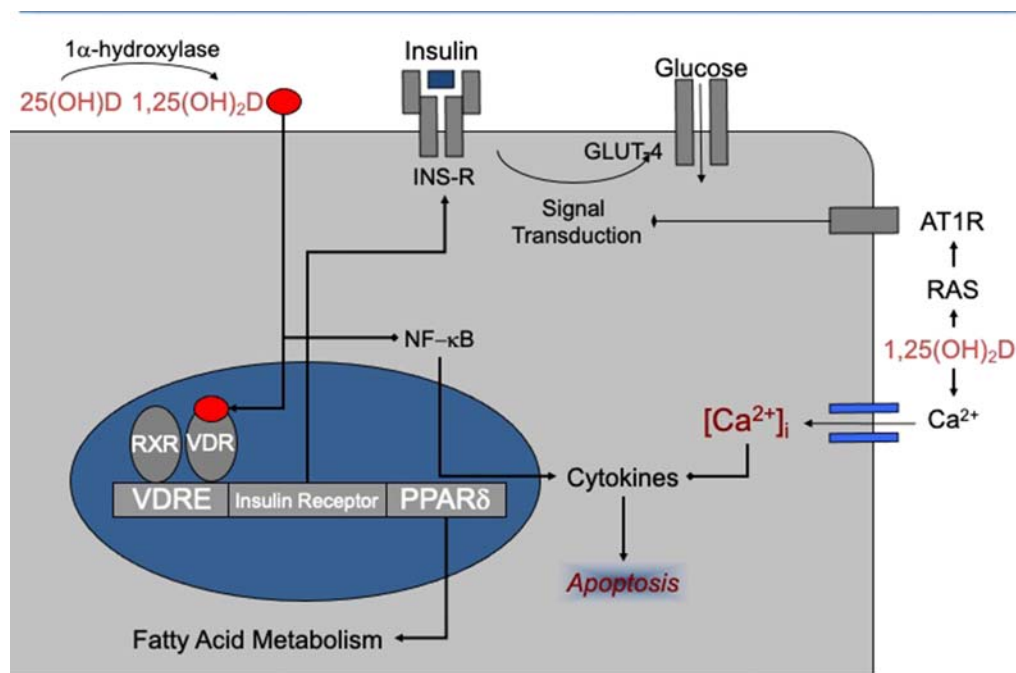
compared with daily doses). In the D2d study, discussed in detail later, vitamin D<sub>3</sub> at 4000 IU/day did not change an OGTT-derived index of beta-cell function in people with prediabetes not selected for vitamin D status; however, there was significant improvement in beta-cell function among those with blood  $25(\text{OH})\text{D}$  below 12 ng/mL [52].

### 3.2 Vitamin D and insulin sensitivity

In peripheral insulin-target cells, active vitamin D metabolites may enhance insulin sensitivity in several ways, as indicated by the presence of the vitamin D receptor in skeletal muscle and adipose tissue (Fig. 76.2) [15,53–57].  $1,25(\text{OH})_2\text{D}$  appears to directly augment insulin sensitivity by stimulating the expression of insulin receptors [58–61];  $1,25(\text{OH})_2\text{D}$  enters insulin-responsive cells and interacts with the VDR, activating the VDR-retinoic acid X-receptor (RXR) complex, which, in turn, binds to a vitamin D response element

found in the human insulin receptor gene promoter. The result is enhanced transcriptional activation of the insulin receptor gene, which increases the total insulin receptors without altering receptor affinity.  $1,25(\text{OH})_2\text{D}$  may also enhance insulin sensitivity by transcriptionally activating peroxisome proliferator-activated receptor delta, a transcription factor implicated in the regulation of fatty acid metabolism in skeletal muscle and adipose tissue [62]. An indirect effect of  $1,25(\text{OH})_2\text{D}$  on insulin sensitivity might also be exerted via its important and well-recognized role in regulating extracellular calcium level and flux through cell membranes. Calcium is known to be essential for insulin-mediated intracellular processes in insulin-responsive tissues such as skeletal muscle and adipose tissue [63,64], with a very narrow range of intracellular calcium needed for optimal insulin-mediated functions [65]. Changes in intracellular calcium in insulin target tissues may contribute to peripheral insulin resistance [65–71] via impaired insulin signal transduction [71,72], leading to decreased glucose transporter





**FIGURE 76.2 Vitamin D and insulin action.** In peripheral insulin-target cells, vitamin D may directly enhance insulin sensitivity in several ways. The active form of vitamin D,  $1,25(\text{OH})_2\text{D}$ , enters the insulin-responsive cells from the circulation and interacts with the vitamin D receptor-retinoic acid x-receptor complex (**RXR-VDR**). The complex binds to a vitamin D response element (**VDRE**), which is found in the human insulin receptor gene promoter, to enhance the transcriptional activation of the insulin receptor gene and increase the synthesis of insulin receptors (**INS-R**) which act to promote glucose uptake via the glucose transporter 4 (**GLUT-4**) receptor and/or by activating peroxisome proliferator-activated receptor delta (**PPAR-δ**), a transcription factor implicated in the regulation of fatty acid metabolism in skeletal muscle and adipose tissue. The effects of vitamin D may be mediated indirectly via regulating extracellular calcium ( $\text{Ca}^{2+}$ ), calcium flux through the cell and intracellular calcium ( $[\text{Ca}^{2+}]_i$ ). Vitamin D may promote beta-cell survival by modulating the generation (through downregulation of nuclear factor- $\kappa\text{B}$  and effects of cytokines). Vitamin D may also affect insulin resistance indirectly through the renin-angiotensin system (**RAS**) via the angiotensin 1 receptor (**AT1R**).

activity [71–73]. Hypovitaminosis D also leads to increased parathyroid hormone (PTH) concentration, which has been associated with increased insulin resistance [74,75]. Vitamin D may also affect insulin resistance indirectly through RAS, as described in the next section. Finally, vitamin D insufficiency is associated with increased fat infiltration in skeletal muscle, independent of body mass, which may further contribute to decreased insulin action [76]. In observational human studies, low vitamin D status (assessed by self-reported vitamin D intake or blood  $25(\text{OH})\text{D}$  level) has been associated with simple indices of insulin resistance, including measurements of fasting insulin and homeostasis model assessment (HOMA-IR) [43–46,77–85], but the association is not consistent [47,81,86,87]. A recent metaanalysis reported no effect of vitamin D supplementation on insulin sensitivity in adults with or at increased risk for insulin resistance [88]; however, this metaanalysis had major limitations, primarily based on the low-quality, short-term follow-up, and heterogeneity of the included studies, including in the definitions of insulin resistance [89].

### 3.3 Vitamin D and systemic inflammation

Systemic inflammation, via an increase in proinflammatory cytokines, plays an important role in the pathogenesis of type 2 diabetes, mostly by promoting insulin resistance; pancreatic beta-cell function may also be affected via cytokine-induced apoptosis [90–92]. Vitamin D can lessen the effects of systemic inflammation on type 2 diabetes risk by altering the balance between pro- and anti-inflammatory cytokines, [93,94].  $1,25(\text{OH})_2\text{D}$  may improve insulin sensitivity and protect against beta-cell cytokine-induced apoptosis by directly modulating the expression and activity of cytokines [41,95–97]. One such pathway may be through downregulation of NF- $\kappa\text{B}$ , which is a major transcription factor for TNF- $\alpha$  and other inflammatory mediators [98,99]. Another pathway that may, at least in part, mediate the antiapoptotic effect of  $1,25(\text{OH})_2\text{D}$  on beta-cells is through counteracting cytokine-induced Fas expression [100]. Several other immune-modulating effects of  $1,25(\text{OH})_2\text{D}$  (e.g., blockade of dendritic cell differentiation, inhibition of lymphocyte proliferation, inhibition of foam cell

formation and cholesterol uptake in macrophages, enhanced regulatory T lymphocyte development) [96,101] may provide additional pathways of protection against inflammation-induced type 2 diabetes risk. Low blood 25(OH)D concentration has been associated with elevated concentration of markers of systemic inflammation in some [84,102–104] but not all human studies [77,105–109].

### 3.4 Other mechanisms

Beyond effects on pancreatic beta-cell function, insulin action, and systemic inflammation, there are other mechanisms of how vitamin D may modulate risk of type 2 diabetes.

It is well established that overweight and obesity are risk factors for vitamin D deficiency typically attributed to a “sequestration” effect of vitamin D in adipose tissue or simply a “volumetric dilutional” effect, due to suboptimal intake, or reduced biosynthesis from limited sunlight exposure [110–112]. Recent evidence shows that obesity represses vitamin D bioactivation by CYP2R1, leading to reduced production of 25(OH)D, while weight loss upregulates CYP2R1 expression [113,114]. These results suggest that the effect of vitamin D on diabetes risk may be mediated via its conversion to 25(OH)D by CYP2R1, which is primarily expressed in the liver [115] and subsequently to 1.25(OH)<sub>2</sub>D by CYP27B1 in the kidney and pancreatic endocrine cells. Therefore, people with obesity are at risk for type 2 diabetes, at least in part, due to their inability to optimize vitamin D activation pathways and their glucose tolerance would be affected the most by vitamin D deficiency.

Other pathways may be relevant. For example, the central nervous system can directly control hepatic glucose production, insulin secretion, and glucose handling by the muscle and kidney [116]. Specifically, the paraventricular nucleus (PVN) is a key hypothalamic area that integrates signals from a variety of brain regions and has important regulatory functions for hepatic glucose control. The PVN may also alter peripheral glucose levels by modifying pancreatic function [116]. Vitamin D is well known to be integral to normal brain development [117], and there is data to support that vitamin D action in the brain is an essential pathway to halt glucose intolerance in diet-induced obese animals [118]. These centrally mediated effects of vitamin D on glucose homeostasis may be mediated by improving insulin action within the hypothalamus or through antiinflammatory actions in the hypothalamus [119,120].

## 4. Observational studies on vitamin D and type 2 diabetes

### 4.1 Seasonal and geographic studies

An association between vitamin D status and type 2 diabetes is suggested by a reported seasonal variation in glycemic control in patients with type 2 diabetes, being worse in the winter [121,122] when hypovitaminosis D is highly prevalent due to decreased exposure to solar UVB light. A strong geographic variability has been described in relation to type 1 diabetes, with incidence rates approaching zero in regions with high UVB irradiance [123–125]; however, a similar geographic association between decreasing UVB exposure and prevalent type 2 diabetes is not seen. Although moderate-level evidence suggests that recreational sun exposure is inversely associated with risk of type 2 diabetes, obesity and lifestyle (diet, exercise), which align with sun exposure, are confounders [126] in the reported relationship.

### 4.2 Case–control and cross-sectional studies

The initial evidence for a role of vitamin D in type 2 diabetes comes from case–control studies, the first of which was published in 1979 [127]. These studies have included small numbers of participants, and most [81,128–138], but not all [81,127,134,139], have reported that patients with type 2 diabetes or glucose intolerance have lower blood 25(OH)D concentration compared with controls without diabetes.

Several large cross-sectional studies have examined the association between vitamin D status (assessed by blood 25(OH)D concentration or self-reported vitamin D intake) and prevalence of glucose intolerance or type 2 diabetes or metabolic syndrome. The latter describes the clustering of several cardiometabolic risk factors (abdominal obesity, hypertension, dyslipidemia defined as high triglycerides and low HDL cholesterol, and hyperglycemia), and it is closely linked to type 2 diabetes [140]. Most studies have reported inverse associations between vitamin D status and glucose intolerance and type 2 diabetes [45,75,78,82,132,141–149], metabolic syndrome [45,83,143,147,149–152], or markers of insulin resistance [46,82], while others did not show any associations [45,75,78,106,142,153–160].

In a large cross-sectional study with data from the US-based National Health Nutrition Examination Survey (NHANES), blood 25(OH)D concentration was inversely associated with prevalence of diabetes in a dose-dependent pattern in non-Hispanic White people and Mexican-American people, after multivariate adjustment, including BMI [78]. In this study, there

was no association in non-Hispanic Black people despite lower 25(OH)D concentration found in this racial group, which may be explained by the observation that people with darker skin complexity exhibit a different vitamin D, calcium, and PTH homeostasis compared with people with lighter skin complexity [161]. A subsequent analysis from NHANES did not find an interaction between blood 25(OH)D concentration and race/ethnicity on glycemic outcomes [82]. Additional studies using NHANES data have repeatedly confirmed the inverse association between blood 25(OH)D concentration and glycemia [82,143,144,146,147], which has also been reported in other large cohorts from the United States [75,149,162], Europe [141,151,152], and China [83].

Vitamin D status has also been inversely associated with the prevalence of the metabolic syndrome in some [45,80,81,83,143,149–152,154,157] but not all studies [75,81,155,163]. In the NHANES database, serum 25(OH)D concentration (after multivariate adjustment) was inversely associated with prevalent metabolic syndrome among both genders and all three major racial or ethnic groups [143,157]. A similar inverse association has also been reported in two cohorts from the United Kingdom [80,151], in a cohort from the Netherlands [152], and in a cohort from China [83]. The clinical components of the metabolic syndrome that have consistently been independently associated with low blood 25(OH)D concentration are abdominal obesity and hyperglycemia [83,152]; therefore, the association between 25(OH)D status and metabolic syndrome reported in these cross-sectional studies may simply reflect the well-established inverse association between 25(OH)D status and adiposity [78,164–166].

### 4.3 Longitudinal cohort studies

Cross-sectional studies cannot establish the direction of causality between vitamin D status and risk of type 2 diabetes; therefore, longitudinal observational studies where vitamin D status is assessed prior to the development of the outcome of interest (incident type 2 diabetes) are methodologically preferred. In most longitudinal observational studies, a significant association between high blood 25(OH)D concentration and lower incidence of type 2 diabetes has been reported [1,154,167–182]; but no association was observed in other studies (Table 76.1) [181,183–188]. These longitudinal studies included participants (nearly all of White race) who were followed from 1.3 to 22 years for incident type 2 diabetes. Six studies included postmenopausal women only [154,167,170,183,184,188]. Studies have assessed vitamin D status by self-reported total vitamin D intake [154,167], predicted 25(OH)D score [169], or by blood 25(OH)D concentration [1,168,170–188]

(Table 76.1A). Ascertainment of type 2 diabetes was by validated self-report [1,154,167,170,186], by laboratory measurement [177–181,186], by combination of self-report and laboratory measurement or validation by participant's physician [169,172,174–176,182–185], by clinical diagnosis [173], or by hospital/national registry-based data [168,171,181].

In the Nurses' Health Study, after multivariate adjustment for age, BMI, and nondietary covariates, women who reported consumption of more than 800 IU/day of vitamin D had a 23% lower risk for developing incident type 2 diabetes compared with women who reported consumption of less than 200 IU/day [167]. The association, however, was attenuated and became non-statistically significant after adjusting for dietary factors. The dietary variables solely responsible for attenuation of the results were magnesium and calcium, which share dietary sources with vitamin D. In the same study, women who reported the highest calcium (>1200 mg/day) and vitamin D (>800 IU/day) intake (1.3% of the cohort) had 33% lower risk of type 2 diabetes compared with women with the lowest calcium (<600 mg/day) and vitamin D (<400 IU/day) intakes, which highlights a potentially important role for calcium intake in type 2 diabetes risk. In the Framingham Offspring Study (men and women), investigators used a subsample of the cohort to develop a regression model to predict blood 25(OH)D concentrations from age, sex, body mass index, month of blood sampling, total vitamin D intake, smoking status, and total energy intake [169]. Using this model, a predicted 25(OH)D score was calculated for each nondiabetic participant, and the association between the predicted 25(OH)D score and incidence of type 2 diabetes was assessed during a mean follow-up period of 6 years. Compared with participants in the lowest tertile of the predicted 25(OH)D score, those in the highest tertile had 40% lower incidence of type 2 diabetes after multivariate adjustment.

Several case–control studies nested within larger cohorts have reported the association between blood 25(OH)D concentration and incident type 2 diabetes [168,170,176,180,181,184] (Table 76.1). In these studies, blood samples from cases (i.e., participants who develop type 2 diabetes) and matched controls (i.e., participants who did not develop diabetes during the follow-up period) are retrieved from stored samples, and blood 25(OH)D is measured at a time when all participants were free of type 2 diabetes. Analyses then compare the 25(OH)D concentration between cases and controls. The study by Knekt et al. which pooled data from two cohorts in Finland (total of 7503 participants), included 412 cases and 986 matched controls [168]. After multivariate adjustment including BMI, participants who were in the highest quartile of 25(OH)D at baseline (mean 25[OH]D 27.6 ng/mL) compared with those in

**TABLE 76.1** Observational longitudinal cohort studies of vitamin D status (plasma or serum 25[OH]D concentration, predicted 25[OH]D concentration, or self-reported vitamin D intake) and new-onset type 2 diabetes.

First author year of publication (country)	Male, %	Mean baseline age (range), year	White race, %	n <sup>a</sup> /N (incidence)	Vitamin D measure; comparison <sup>b</sup>	Mean follow-up, year (start-end)	Results, adjusted RR, OR, or HR (95% CI) <i>P</i> for trend	Outcome (ascertainment method)	Adjustments	Study quality <sup>c</sup>
Liu (2005) Women's health study (US)	0	52 (45–75)	95	805/10,066 (8%); cohort study	Vitamin D intake (total); ≥511 versus ≤159 IU/day	9 (ND)	0.73 (0.54, 0.99) <sup>d</sup>	Type 2 diabetes (validated self-report)	Age	Fair
Pittas (2006) Nurses health study (US)	0	46 (30–55)	98	4,843/83,779 (5.8%); cohort study	Vitamin D intake (total); >800 versus ≤200 IU/day	20 (1980–2000)	0.87 (0.69, 1.09)	Type 2 diabetes (validated self-report)	Age, BMI, exercise, residence, and others <sup>e</sup>	Fair
Mattila (2007) Mini-Finland health survey (Finland)	47	53 (40–69)	100	187/4,097 (4.6%); cohort study	25(OH)D; >22 versus <12 ng/mL	17 (1978–24)	0.60 (0.36–0.98)	Type 2 diabetes (nationwide registry of patients receiving medication reimbursement)	Age, sex, and the month when the blood samples were collected	Fair
Knekt (2008) Finnish mobile clinic health examination survey (Finland)	100	ND (40–74)	100	105/1,628 (6.4%); nested case–control study with 206 controls	25(OH)D; 30 versus 10 ng/mL (means)	22 (1973–94)	0.49 (0.15, 1.64)	Type 2 diabetes (medication-treated, registry-based)	Age, BMI, exercise, season, and others <sup>f</sup>	Good
	0	ND (40–74)	100	125/1699 (7.4%); nested case–control study with 246 controls	25(OH)D; 25 versus 9 ng/mL (means)		0.91 (0.37, 2.23)		Age, BMI, exercise, season, and others <sup>f</sup>	
Knekt (2008) Mini-Finland health survey (Finland)	100	53 (40–69)	100	83/1,948 (4.3%); nested case–control study with 245 controls	25(OH)D; 31 versus 9 ng/mL (means)	17 (1978–94)	0.17 (0.05, 0.52)	Type 2 diabetes (medication-treated, registry-based)	Age, BMI, exercise, season, and others <sup>f</sup>	Good
	0	ND (40–69)	100	99/2,228 (4.4%); nested case–control study with 289 controls	25(OH)D; 25 versus 8 ng/mL (means)		1.45 (0.58, 3.62)		Age, BMI, exercise, season, and others <sup>f</sup>	
Anderson (2010) Intermountain healthcare system (US)	25	55 (NR)	86	913/41,504 (2.2%); cohort study	25(OH)D; >30 versus ≤15 ng/mL	1.3 (mean), (2000–09)	0.53 (0.43–0.65)	Type 2 diabetes (clinical diagnosis)	Age, gender, history of hypertension, hyperlipidemia, diabetes mellitus, peripheral vascular disease, and other clinical conditions	Fair

Continued



**TABLE 76.1** Observational longitudinal cohort studies of vitamin D status (plasma or serum 25[OH]D concentration, predicted 25[OH]D concentration, or self-reported vitamin D intake) and new-onset type 2 diabetes.—cont'd

First author year of publication (country)	Male, %	Mean baseline age (range), year	White race, %	n <sup>a</sup> /N (incidence)	Vitamin D measure; comparison <sup>b</sup>	Mean follow-up, year (start-end)	Results, adjusted RR, OR, or HR (95% CI) P for trend	Outcome (ascertainment method)	Adjustments	Study quality <sup>c</sup>
Bolland (2010) (New Zealand)	0	74 (>55 years)	ND	29/1,471 (2%); postmenopausal women; cohort study	25(OH)D; ≥20 versus <20 ng/mL	4.6 (1998–2003)	0.9 (0.4–1.9)	Type 2 diabetes (self-reported, verified using medical records)	Age, body weight, smoking status, treatment allocation (calcium or placebo), and season	Fair
Grimnes (2010) Tromsø study (Norway)	64	58 (50–74)	100	183/4,157 nonsmokers (4.4%); 64/1,962 smokers (3.1%); cohort studies	25(OH)D; <ul style="list-style-type: none"> <li>• Nonsmokers: 29 versus 14 ng/mL</li> <li>• Smokers: 39 versus 29 ng/mL (means)</li> </ul>	11 (1994–2005)	Nonsmokers: 0.73 (0.48–1.12) Smokers: 0.68 (0.29–1.61)	Type 2 diabetes (self-reported questionnaire, glycemic status, and hospital-confirmed diagnosis)	Age, sex, BMI, physical activity, number of cigarettes smoked, and years of smoking (in current smokers)	Fair
Liu (2010) Framingham offspring study (US)	54	60	~100	133/2,956 (4.4%); cohort study	Predicted 25(OH) D score; 22 versus 17 ng/mL (median)	7 (1991–2001)	0.60 (0.37–0.97)	Type 2 diabetes (medication-treated, laboratory-based)	Age, sex, and waist circumference <sup>g</sup>	Fair
Pittas (2010) Nurses' health study (US)	0	56 (43–70)	98	608/32,826 (1.8%); nested case-control study with 559 controls	25(OH)D; 33 versus 14 ng/mL (median)	14 (1990–2004)	0.52 (0.33–0.83)	Type 2 diabetes (validated self-report)	Age, BMI, exercise, season, race, and others <sup>h</sup>	Good
Gagnon (2011) AusDiab study (Australia)	45	50 (NR)	>90	199/5,200 (3.8%); cohort study	25(OH)D; 37 versus 16 ng/mL (Median)	5 (1999–2004)	0.56 (0.36–0.86)	Type 2 diabetes (treatment, fasting glucose, or 2-h OGTT)	Age, ethnicity, waist circumference, family history of diabetes, smoking status, physical activity, and season and latitude	Fair
Kayanil (2011) PROspective metabolism and ISlet cell evaluation (PROMISE) (Canada)	27	50 (>30)	61	30/489 (6.1%); cohort study	25(OH)D; ≥20 versus <12 ng/mL	3 (2004–06)	0.69 (0.53–0.89)	Type 2 diabetes (self-report and verification from the participant's physician through a supplementary form requesting information)	Age, sex, ethnicity, season, and baseline value of the outcome variable, as well as baseline and change in physical activity, vitamin D	Fair

Robinson (2011) The women's health initiative (US)	0	66 (50–79)	90	317/5,140 (6.2%); nested case –control study with 4823 controls	25(OH)D; ≥30 versus <20 ng/mL	7.3 (1993–2004)	1.05 (0.62–1.76)	Type 2 diabetes (self-report of physician diagnosis or receiving insulin or oral hypoglycemic medication)	supplement use, and BMI Age, ethnicity, latitude, month of blood draw, WHI ancillary study indicators, BMI, hypertension, fiber intake, magnesium intake, and physical activity	Fair
Thorand (2011) The MONICA/ KORA augsburg study (German)	53	54 (35–74)	ND	416/7,936 (5.2%); nested case –control study with 1267 controls	25(OH)D; Men: 27 versus 11 ng/mL; Women: 23 versus 10 ng/mL (median)	11 (1984–2002)	0.63 (0.44–0.90)	Type 2 diabetes (questionnaires or interviews validated by physician or medical chart review)	Age, sex, survey, season, BMI, smoking status, alcohol intake, physical activity, systolic BP, total cholesterol/ HDL-cholesterol, and parental history of diabetes	Fair
Pittas (2012) The diabetes prevention program (US)	33	51 (≥25)	57	426/2,039 (20.1%); cohort within a randomized trial	25(OH)D; 30 versus 13 ng/mL (median)	2.7 (1996–2001)	0.72 (0.56–0.90)	Type 2 diabetes (OGTT)	Recruitment location, age, sex, BMI, race, physical activity, family history of diabetes, history of hypertension, smoking status, alcohol consumption, multivitamin use, CRP, kidney function, calcium intake, and treatment arm (intensive lifestyle or placebo)	Good
Deleskog (2012) The stockholm diabetes	59	48 (35–56)	ND	279/2,378 (11.7%); nested case–control	25(OH)D; >28 versus <18 ng/mL	8–10	Men: 0.52 (0.30 –0.90)	Type 2 diabetes (OGTT)	Age, BMI, family history of diabetes, physical	Fair

Continued

**TABLE 76.1** Observational longitudinal cohort studies of vitamin D status (plasma or serum 25[OH]D concentration, predicted 25[OH]D concentration, or self-reported vitamin D intake) and new-onset type 2 diabetes.—cont'd

First author year of publication (country)	Male, %	Mean baseline age (range), year	White race, %	n <sup>a</sup> /N (incidence)	Vitamin D measure; comparison <sup>b</sup>	Mean follow-up, year (start-end)	Results, adjusted RR, OR, or HR (95% CI) P for trend	Outcome (ascertainment method)	Adjustments	Study quality <sup>c</sup>
prevention program (Sweden)				study with 1011 controls			Women: 0.79 (0.36–1.73)		activity, and blood pressure	
Forouhi (2012) European prospective investigation into cancer (EPIC)-Norfolk study (UK)	42	58 (40–75)	99	621/25,639 (2.4%); nested case-cohort study with 826 controls	25(OH)D; >32 versus <20 ng/mL	9–13 (1993–2006)	0.50 (0.32–0.76)	Type 2 diabetes (medical record linkage with general practice, hospital, and death registries)	Age, sex, season, BMI, family history of diabetes, smoking, physical activity, education, alcohol intake, and supplement and/or cod liver oil use	Fair
Forouhi (2012) The Medical Research Council (MRC) Ely study (UK)	42	64 (40–69)	99	37/777 (4.8%); cohort study	25(OH)D; 24 versus 23 ng/mL (means)	10 (1990–2003)	0.69 (0.17–2.91)	Type 2 diabetes WHO criteria (OGTT)	Age, sex, season, BMI, family history of diabetes, alcohol intake, smoking, socioeconomic status, and physical activity	Fair
Gonzalez- Molero (2012) Pizarra study (Southern Spain)	43	50	ND	26/412 (6.3%); cohort study	25(OH)D; >18 versus ≤18 ng/mL	4 (1996–2007)	0.17 (0.05–0.61)	Type 2 diabetes (OGTT and glycosylated hemoglobin)	Age, sex, obesity (BMI>30), smoking, outdoor activity, alcohol intake, month of blood sampling, PTH, phosphorus, and creatinine	Fair
Husemoen (2012) Inter99 study (Denmark)	48	46 (30–65)	~100	141/4,296 (3.3%); cohort within a randomized trial	25(OH)D; ≥30 versus <10 ng/mL	5 (1999–2006)	0.61 (0.28–1.33)	Type 2 diabetes (OGTT, glycosylated hemoglobin, known history of diabetes, and/or use of diabetes medication)	Age, sex, BMI, season of blood collection, family history of diabetes, change in weight during the follow-up, physical activity, dietary habits, alcohol intake,	Good

									smoking status, total energy intake, social class, and randomization group and self-reported changes in dietary habits, physical activity, smoking status, and alcohol intake during the follow-up	
Husemoen (2012) MONICA 1 population survey (Denmark)	50	55 (41–71)	~100	288/2,571 (11.2%); cohort study	25(OH)D; $\geq 30$ versus $<10$ ng/mL	16 (1993–2010)	0.57 (0.38–0.85)	Type 2 diabetes (information on hospitalization, diabetes registration, blood glucose levels, or antidiabetic medication use)	Age, sex, season of blood collection, history of CVD, family history of diabetes, waist circumference, physical activity, healthy food index, fish intake, supplement use, smoking status, alcohol intake, and educational level	Fair
Pilz (2012) The Hoorn study (Austria)	49	68 (50–75)	ND	45/280 (16%); cohort study	25(OH)D; $\geq 30$ versus $<20$ ng/mL	7.5 (2000–09)	0.52 (0.13–2.10)	Type 2 diabetes (OGTT, glycosylated hemoglobin, and/or hypoglycemic drugs)	Age, sex, season, BMI, physical activity, hypertension, fasting glucose, HDL-C, and triglycerides	Fair
Afzal (2013) Copenhagen city heart study (Denmark)	44	56 (20–100)	100	810/9,841 (8.2%); cohort study	25(OH)D; $\geq 20$ versus $<5$ ng/mL	20 (1981–2010)	1.35 (1.09–1.66)	Type 2 diabetes (self-reported and use of antidiabetic medicine)	Sex, age, smoking status, body mass index, income, physical activity, HDL-cholesterol, and calendar month of blood draw	Fair
Buijsse (2013) The European	43	62 (35–65)	ND			6.6 (1994–2008)	0.98 (0.89–1.08)	Type 2 diabetes (self-reported)	Age, sex, center, season of blood	Fair

Continued



**TABLE 76.1** Observational longitudinal cohort studies of vitamin D status (plasma or serum 25[OH]D concentration, predicted 25[OH]D concentration, or self-reported vitamin D intake) and new-onset type 2 diabetes.—cont'd

First author year of publication (country)	Male, %	Mean baseline age (range), year	White race, %	n <sup>a</sup> /N (incidence)	Vitamin D measure; comparison <sup>b</sup>	Mean follow-up, year (start-end)	Results, adjusted RR, OR, or HR (95% CI) <i>P</i> for trend	Outcome (ascertainment method)	Adjustments	Study quality <sup>c</sup>
prospective investigation into cancer and nutrition (EPIC) (German)				1,572/3,693 (42.6%); cohort study	25(OH)D; ≥20 versus <10 ng/mL				draw, education, and lifestyle	
Schottker (2013) ESTHER study (German)	42	62 (50–74)	ND	829/7,791 (10.6%); cohort study	25(OH)D; <12 versus ≥20 ng/mL	7.9 (2000–10)	1.38; 1.09–1.75	Type 2 diabetes (self-reported validated by standardized questionnaires)	Age, sex, month of blood draw, regular intake of multivitamin supplements, fish consumption, school education, physical activity, smoking, SBP, CRP, total cholesterol, and CKD	Fair
Tohidi (2013) Tehran lipid and glucose study (Iran)	NA	50 (20–83)	NA	191/761 (25%); nested case–control study with 570 controls	25(OH)D; >22 versus 3–11 ng/mL	3.6	0.73 (0.74–1.13)	Type 2 diabetes (ADA Criteria, 2003; antidiabetic drugs, FG, or 2-h OGTT)	Age, sex, family history of diabetes, baseline SBP, triglyceride-to-HDL ratio, waist-to-height ratio, leisure-time physical activity, lifestyle modification intervention, and FG	Fair
Abbas (2014) EPIC-InterAct study (German)	NA	53	NA	11,994/27,043 (44.3%); nested case–cohort study with 15,798 controls	Vitamin D intake (total); ≥243 versus ≤87.6 IU/day	10.8	1.09 (0.97–1.22)	Type 2 diabetes self-reports (history of diabetes, physician-diagnosed diabetes, antidiabetic drug use), primary and secondary care registers, drug registers, hospital	BMI, physical activity, education level, smoking status, and alcohol intake	Fair

Schafer (2014) study of osteoporotic fractures (US)	0	77 ( $\geq 65$ )	~100	320/5,463 (6%); cohort study	25(OH)D; $\geq 30$ versus $<20$ ng/ mL	8.6 (1986–2008)	0.88 (0.64–1.20)	admissions and mortality data, and individual medical record reviews Type 2 diabetes (self-reported)	Age and clinic site; BMI, self- reported health, and hypertension	Fair
Veronese (2014) Progetto Veneto Anziani (Pro.V.A.) study (Italy)	41	76 (65–103)	~100	291/2,227 (13%); cohort study	25(OH)D; $\geq 30$ versus $<10$ ng/ mL	4.4	1.44 (0.95–1.98)	Type 2 diabetes (questionnaires or interviews validated by physician)	Age and gender	Fair

<sup>a</sup>Number of cases if nested case–control study.

<sup>b</sup>Highest/lowest risk category versus reference category.

<sup>c</sup>Study quality is determined based on a three-category grading system. Good quality studies adhere most closely to the commonly held concepts of high quality including clear descriptions of the population and setting; unbiased assessments of vitamin D status and outcomes; appropriate statistical analysis including multivariable analysis adjusting for age, race, weight, and sun exposure and intention-to-treat analysis; no obvious reporting omissions or errors; and  $<20\%$  dropouts. Fair quality studies have some deficiencies in the above criteria, but these are unlikely to cause major bias. Studies that used vitamin D intake or predicted 25(OH)D score as the predictor are rated fair. Poor quality studies have major deficiencies in design, analyses, or reporting, such that major bias could not be excluded.

<sup>d</sup>Estimated from reported data.

<sup>e</sup>Family history of diabetes, hypertension, calcium intake, smoking, alcohol, coffee, and other diet.

<sup>f</sup>Smoking, education, and medications.

<sup>g</sup>Family history of diabetes, hypertension, low HDL-cholesterol, high triglycerides, impaired fasting glucose, and diet.

<sup>h</sup>Fasting status, latitude, hypercholesterolemia, hypertension, family history of diabetes, smoking, physical activity, alcohol, multivitamin use, and diet.

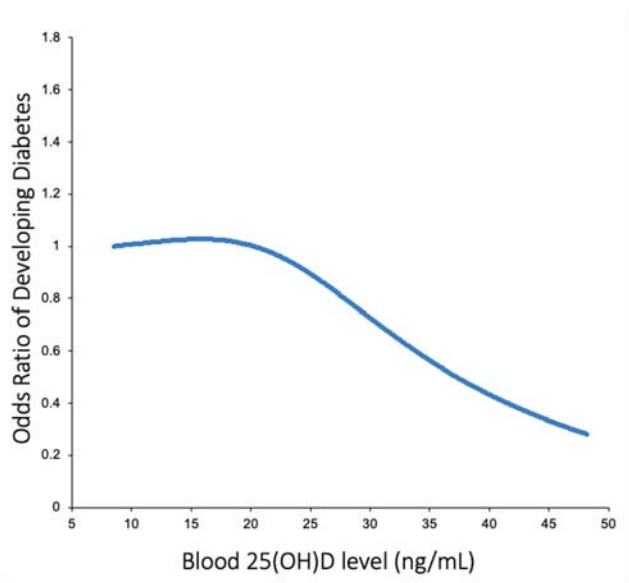
25(OH)D, plasma or serum 25-hydroxyvitamin D; BMI, body mass index; BP, blood pressure; FG, fasting glucose; HR, hazard ratio; IU, international units; ND, no data; OGTT, oral glucose tolerance test; OR, odds ratio; RR, relative risk. To convert 25(OH)D concentration from ng/mL to nmol/L multiply by 2.459.

the lowest quartile (mean 25[OH]D 8.9 ng/mL) had 40% lower risk of developing type 2 diabetes. The result was entirely driven by a lower risk among men only while there was no significant association among women. Another nested case–control study by Pittas et al. was conducted in the Nurses' Health Study among 608 women with newly diagnosed type 2 diabetes and 559 matched controls [170]. After adjusting for matching factors and diabetes risk factors, including BMI, the odds ratio for incident type 2 diabetes in the top (median 25 [OH]D 33.4 ng/mL) versus the bottom (median 25 [OH]D 14.4 ng/mL) quartile of 25(OH)D was 0.52 (95% confidence interval, 0.33, 0.83). The association was consistent across subgroups of baseline BMI, age, and calcium intake although the association appeared to be stronger among overweight/obese versus normal-weight women. In the same study, spline regression models showed no apparent threshold and no deviation from linearity for the relation between 25(OH)D and risk of incident type 2 diabetes, although the shape of the figure suggested a stronger decrease in risk within the higher range of plasma 25(OH)D concentration, above 30–35 ng/mL (Fig. 76.3). Three more nested case–control studies from Germany [176], Sweden [180], and the United Kingdom [181] also showed strong inverse associations between high levels of blood 25(OH)D and incident type 2 diabetes. A post-hoc analysis of three nested case-control studies of fractures, colon cancer, and breast cancer that measured blood 25(OH)D at baseline in women participating in the

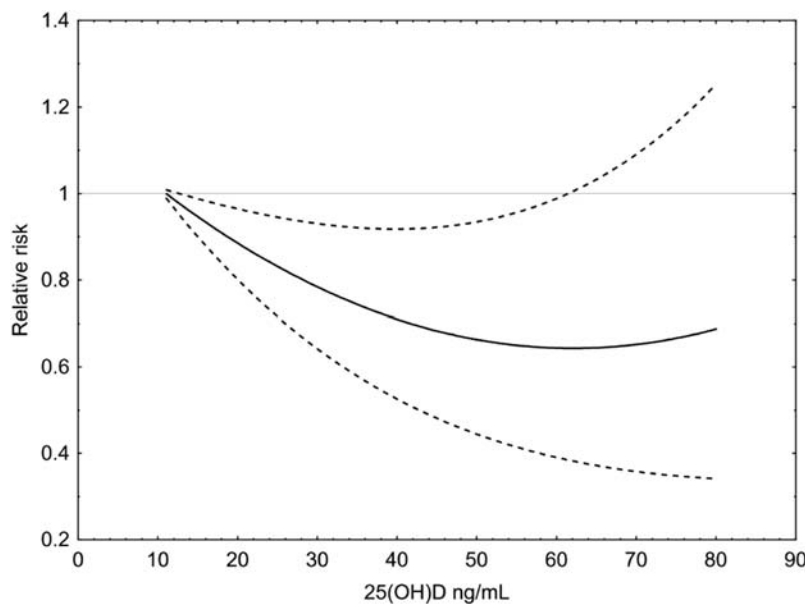
WHI study reported no association among older postmenopausal women [184].

In addition to the nested case–control studies, several longitudinal cohort studies have been published, and nearly all of them have reported inverse associations between blood 25(OH)D concentration and incident type 2 diabetes (Table 76.1). Several metaanalyses have summarized results from the observational studies. In a study by Song et al. which combined data from a total of 21 longitudinal observational studies (76,220 participants and 4996 incident type 2 diabetes cases), there was a significant favorable association between 25(OH)D concentration and incident type 2 diabetes (risk ratio 0.62; 95% confidence interval [CI] 0.54–0.70 for the highest vs. lowest 25[OH]D concentration) [189]. A spline regression model showed that higher 25(OH)D levels were monotonically associated with a lower diabetes risk. This inverse association did not differ by sex, duration of follow-up, study sample size, diabetes diagnostic criteria, or 25(OH)D assay method. In another metaanalysis that included data from 22 longitudinal studies (8492 cases and 89,698 noncases), one standard deviation (10 mg/dL) lower 25(OH)D concentration was associated with an increased risk of type 2 diabetes with moderate evidence of heterogeneity [190]. In a more recent metaanalysis that combined data from 28 longitudinal studies, there was a 34% lower risk of type 2 diabetes (risk ratio 0.66; 95% confidence interval) when comparing individuals with the highest category of 25(OH)D with those in the lowest [191]. In the latest metaanalysis of large longitudinal observational studies, a 1–standard deviation higher level of total 25(OH)D was associated with 20% lower risk of type 2 diabetes [192].

Nearly all observational cohort studies have examined the association between a single 25(OH)D measurement at baseline and risk of developing type 2 diabetes; however, a single 25(OH)D measurement may not reflect long-term vitamin D status during follow-up. A longitudinal observational study by Pittas et al. examined the association between blood 25(OH)D level, measured annually, and risk of incident type 2 diabetes among participants with prediabetes who were randomized to the intensive lifestyle or placebo arms of the Diabetes Prevention Program [193]. By measuring 25(OH)D at multiple time points, this study obtained an integrated measure of intratrial vitamin D status for each participant, rather than relying on a single baseline value, as all other observational studies have done. After multivariate adjustment, including for the Diabetes Prevention Program lifestyle intervention, there was an inverse association between intra-study blood 25(OH)D level and incident type 2 diabetes. The hazard ratio for diabetes in the top tertile of blood 25(OH)D (mean 30 ng/mL) versus the bottom tertile (mean 13 ng/mL)



**FIGURE 76.3** In the Nurses' Health Study (healthy women at average risk for diabetes), there was a progressively lower risk of developing type 2 diabetes with increasing concentration of blood 25(OH)D without an apparent plateau.



**FIGURE 76.4** Dose–response relationship between blood 25(OH)D concentrations and the relative risk for type 2 diabetes mellitus. Results of restricted cubic splines analysis of relative risks standardized to a common reference category with 12 ng/mL 25(OH)D as midpoint and inverse variance weights. Dashed lines indicate 95% confidence. From Figure 6 in Ref. [191].

was 0.72 (95% confidence interval 0.56–0.90). As in the analysis in the Nurses’ Health Study, there was no apparent threshold for the relation between 25(OH)D level and incident diabetes, and results suggested a stronger decrease in diabetes risk for 25(OH)D levels above >50 ng/mL although very few participants achieved that level.

Findings in the Nurses’ Health Study and Diabetes Prevention Program cohorts are fully concordant with those from other observational longitudinal studies that have reported on the association between higher blood 25(OH)D and lower risk of developing type 2 diabetes. When combining such data in a metaanalysis, results mirrored those from the Nurses’ Health Study and Diabetes Prevention Program cohorts: higher 25(OH)D levels (>40 ng/mL) conferred lower risk of developing diabetes, with no apparent plateau for benefit up to concentrations of about 65 ng/mL (Fig. 76.4) [189,191].

#### 4.4 Mendelian randomization studies

Observational studies using Mendelian randomization approaches have shown mixed results with most of them reporting no associations between genetically predicted blood 25(OH)D concentration and new-onset type 2 diabetes [187,190,194–196], while others reporting inverse associations between higher 25(OH)D levels and new-onset type 2 diabetes [197–199]. The theoretical advantage of the Mendelian randomization studies, which examine the association between genetic variants (single-nucleotide polymorphism) that influence blood 25(OH)D concentration and risk of type 2 diabetes, is

that the measured association with the outcome of interest is less likely to be affected by confounding or reverse causation, which are common challenges in conventional observational studies. However, Mendelian randomization studies center upon assumptions that are not relevant to vitamin D physiology [190]. For example, the alleles included in genetically predicted 25(OH)D level account for less than 5% of the variance on blood 25(OH)D level, which is not surprising given that 25(OH)D concentration is primarily a function of exposure to UVB light and to a lesser degree, dietary intake and supplementation. In general, these studies have examined genetic variants shown to be associated with 25(OH)D concentration and implicated in the synthesis, transport, and metabolism of vitamin D: DHCR (encoding 7-dehydrocholesterol reductase enzyme for synthesis of previtamin D<sub>3</sub>), CYP2R1 (encoding the 25-hydroxylase enzyme for conversion of vitamin D<sub>3</sub> to 25[OH]D), GC (the group-specific component that encodes vitamin D-binding protein), and CYP24A1 (encoding the 24-hydroxylase involved in the clearance of 25[OH]D). One potential limitation is that many of these gene variants have pleiotropic effects beyond determining 25(OH)D concentration. In studies that used the two “synthesis” variants that are specific to 25(OH)D and do not have pleiotropic effects, higher genetically determined 25(OH)D levels were associated with reduced risk of type 2 diabetes [198,199]. In one study among Chinese participants, a 10 ng/mL higher genetically determined 25(OH)D concentration based on “synthesis” variants only was associated with a 14% lower risk of diabetes [198]. In another study among individuals of European ancestry, for 1-standard deviation increment of



genetically determined 25(OH)D concentration, there was a 10% lower risk of developing type 2 diabetes based on three variants within or near genes involved in vitamin D synthesis [199]. Furthermore, Mendelian randomization studies cannot predict amounts of bioavailable vitamin D and cannot distinguish between endogenous and exogenous vitamin D. Indeed, variation in the DHCR7 gene, which is related to endogenous production of vitamin D, has been associated with type 2 diabetes risk [197]. There is also little correlation between 25(OH)D (the predictor in Mendelian randomization studies) and the active form of vitamin D ( $1,25[\text{OH}]_2\text{D}$ ). Another limitation of Mendelian randomization studies is that they are limited to participants of European origin, which limits transferability of findings to other racial and ethnic groups. Another explanation why Mendelian randomization studies have not reported consistent associations between genetically predicted 25(OH)D concentration and type 2 diabetes is because they have assumed linear dose–response relationships. Conventional observational analyses have reported nonlinear associations between measured blood 25(OH)D level and new-onset diabetes with the highest risk being among those with the lowest 25(OH)D levels; therefore, standard Mendelian randomization methods that assume linearity provide an incomplete picture of physiology. Promising new approaches in Mendelian randomization analyses allow for the study of nonlinear associations between the exposure and outcome. For example, in two recent publications that did stratified Mendelian randomization analyses in population subgroups with different 25(OH)D concentrations, inverse causal relationships were uncovered between genetically predicted 25(OH)D and all-cause mortality [200] and cardiovascular disease [201]. However, the methods applied by the investigators in this analysis have been questioned. In summary, results from Mendelian randomization studies should be interpreted cautiously because they may lead to unwarranted conclusions of no association with type 2 diabetes [190].

#### 4.5 Limitations of observational studies and the issue of confounding

Overall, the evidence from the mechanistic studies and human observational and ecologic studies suggests an association between low vitamin D status and risk of type 2 diabetes. However, definite conclusions cannot be drawn solely based on this evidence for a variety of reasons: (1) Ecologic studies provide interesting data and assist in generating hypotheses; however, they are likely to be confounded by many variables. For example, worsening glycemic control in the winter may be due to physical inactivity and weight gain, which are common in the winter months. (2) In cross-sectional or case–control

studies, vitamin D was measured in patients with glucose intolerance or established diabetes; therefore, these measures may not reflect vitamin D status prior to diagnosis and, as a result, the causative nature of the reported associations cannot be established. (3) There is considerable variability among the various cohorts (normal glucose tolerance versus diabetes [newly diagnosed vs. established], age, ethnicity, latitude, mean vitamin D status, etc.), which makes it difficult to compare, contrast, and combine results. (4) In many observational studies, there is lack of adjustment for important confounders.

The most important limitation of the observational studies is residual confounding, which may explain, at least in part, the inverse association between vitamin D status and risk of type 2 diabetes reported in nearly all observational studies. Vitamin D status is an excellent marker of good health, as high 25(OH)D concentration is associated with young age, normal body weight, and a healthy lifestyle, including good dietary and exercise behaviors. A lower vitamin D status may reflect chronic nonspecific illness, which may prevent individuals from engaging in outdoor activities and sun exposure. Vitamin D is also associated with smoking, parental history of cardiovascular disease, and less alcohol intake [202], which are all risk factors for type 2 diabetes. Additional issues related to food synergy may further complicate the study of the association between vitamin D and type 2 diabetes [203]. For example, a higher vitamin D intake is often associated with higher intake of a certain food groups (e.g., dairy) or an overall “prudent” dietary pattern; therefore, additional components in foods that are consumed with vitamin D may have direct effects on type 2 diabetes or, alternatively, foods rich in vitamin D may replace other foods that increase risk of these conditions. Mendelian randomization studies have attempted to address confounding; however, as noted earlier, Mendelian randomization studies involve many assumptions that may not be relevant in the study of vitamin D and type 2 diabetes risk.

### 5. Vitamin D supplementation in people with normal glucose tolerance or type 2 diabetes

Several trials have reported the effect of vitamin D (ergocalciferol [ $\text{D}_2$ ] or cholecalciferol [ $\text{D}_3$ ] or eldcalcitol [an analog of calcitriol, the active form of vitamin  $\text{D}_3$ ]) supplementation (with or without calcium) on various outcomes related to diabetes, including changes in glycemic measures, insulin sensitivity, beta-cell function, development of diabetes, or regression to normal glucose regulation from prediabetes (Table 76.2). Trials have included participants with normal glucose tolerance, at risk for diabetes, prediabetes (based on glycemic measures), or established type 2 diabetes (early stage or

**TABLE 76.2** Randomized controlled trials of the effect of vitamin D (with or without calcium) on glucose tolerance in adults with normal glucose tolerance or established type 2 diabetes.

First author year of publication (country)	Mean baseline age (range), y	Target population	Baseline 25(OH)D, ng/mL	Interventions (number of participants)	Study duration	Effect of vitamin D versus placebo/control	Study quality
<i>Populations at average risk for type 2 diabetes or with normal glucose tolerance</i>							
Nilas (1984) (Denmark)	ND (45–54)	Healthy postmenopausal women	ND	D <sub>3</sub> 2,000 IU/day (n = 25) versus placebo (n = 103). All received calcium, 500 mg/day	2 years	↔FG	Fair
Pittas (2007) (US)	71 (≥65)	Normal fasting glucose	30	D <sub>3</sub> 700 IU/day plus calcium citrate 500 mg/day (n = 108) versus placebo (n = 114)	3 years	↔FG ↔HOMA-IR	Fair
	71 (ND)	Prediabetes (IFG)	30	D <sub>3</sub> 700 IU/day plus calcium citrate 500 mg/day (n = 45) versus placebo (n = 47)	3 years	↓FG ↓HOMA-IR	
De Boer (2008) (US)	62 (50–79)	Healthy postmenopausal women without diabetes	<32	D <sub>3</sub> 400 IU/day plus calcium carbonate 1,000 mg/day (n = 16,999) versus placebo (n = 16,952)	7 years	↔Incidence of diabetes (self-reported)	Good
	ND (50–79)	Healthy postmenopausal women with normal glucose tolerance		D <sub>3</sub> 400 IU/day plus calcium carbonate 1,000 mg/day (n = 738) versus placebo (n = 771)	6 years	↔FG ↔HOMA-IR	
	ND (50–79)	Healthy postmenopausal women with prediabetes (IFG)		D <sub>3</sub> 400 IU/day plus calcium carbonate 1,000 mg/day (n = 718) versus placebo (n = 739)	6 years	↔FG ↔HOMA-IR	
Avenell (2009) (UK)	77 (≥70)	History of fracture	ND	D <sub>3</sub> 800 IU/day (n = 2,649) versus placebo (n = 2,643) (2 × 2 factorial design with calcium carbonate 1,000 mg/day)	2–5 years	↔Incidence of diabetes (self-reported)	Good
Nagpal (2009) (India)	43 (>35)	Healthy men with central obesity	15	D <sub>3</sub> 120,000 IU three times (~8,571 IU/day) (N = 35) versus placebo (N = 36)	6 weeks	↑Oral glucose insulin sensitivity (OGIS) ↔HOMA-IR ↔QUICKI	Fair

Continued

**TABLE 76.2** Randomized controlled trials of the effect of vitamin D (with or without calcium) on glucose tolerance in adults with normal glucose tolerance or established type 2 diabetes.—cont'd

First author year of publication (country)	Mean baseline age (range), y	Target population	Baseline 25(OH)D, ng/mL	Interventions (number of participants)	Study duration	Effect of vitamin D versus placebo/control	Study quality
Zittermann (2009) (Germany)	48 (18–70)	Healthy overweight (BMI>27 kg/m <sup>2</sup> )	12	D <sub>3</sub> 3,332 IU/day (n = 100) versus placebo (n = 100). All received weight reduction advice for 24 weeks	1 year	↔ HbA1c ↔ FG	Good
Von Hurst (2009) (New Zealand)	42 (23–68)	Women with insulin resistance and 25(OH)D < 20 ng/mL	8	D <sub>3</sub> 4,000 IU/day (n = 42) versus placebo (n = 39)	26 weeks	↔ FG ↓ HOMA-IR	Fair
Jorde (2010) (Norway)	ND (21–70)	Overweight/obese	23	D <sub>3</sub> 40,000 IU/week (~5,714 IU/day) (n = 150) versus D <sub>3</sub> 20,000 IU/week (~2,857 IU/day) (n = 139) versus placebo (n = 149). All received calcium 500 mg/day	1 year	↔ HbA1c ↔ FG ↔ 2 hG ↔ HOMA-IR ↔ QUICKI	Fair
Beilfus (2012) (Norway)	50 (23–70)	Healthy, overweight/obese	23	D <sub>3</sub> 40,000 IU/week (n = 110); D <sub>3</sub> 20,000 IU/week (n = 110); versus placebo (n = 112). All received calcium 500 mg/day	1 year	No glycemic data ↔ HOMA-IR ↔ QUICKI	Fair
Wood (2012) (US)	64 (60–70)	Healthy, postmenopausal women	14	D <sub>3</sub> 1,000 IU/day (n = 101) versus D <sub>3</sub> 400 IU/day (n = 102) versus placebo (n = 102)	1 year	↔ FG ↔ HOMA-IR	Fair
Mitchell (2015) (US)	28 (18–45)	Healthy and 25(OH)D < 20 ng/mL	18	D <sub>3</sub> 50,000 IU/week (~7,140 IU/day) (n = 40) versus placebo (n = 50)	12 weeks	↔ FG ↔ HOMA-IR ↔ Insulin secretion; insulin sensitivity	Fair
Hel-Hajj Fuleihan (2016) (Lebanon)	71	Healthy, overweight (69% with prediabetes)	20	D <sub>3</sub> 10,000 IU/Week (n = 110) versus placebo (n = 112). All received calcium 1,000 mg/day and D <sub>3</sub> 500 IU/day	1 year	↔ HbA1c ↔ FG (6 vs. 3 mg/dL; P < .05) ↔ HOMA-IR, lnHOMA	Fair
Tepper (2016) (Israel)	47 (20–65)	Healthy men and 25(OH)D < 20 ng/mL	16	D <sub>3</sub> 100,000 IU/bimonthly (~666 UI/day) (n = 78) versus placebo (n = 52)	1 year	↔ FG ↓ HOMA-IR	Fair

Mousa (2017) (New Zealand)	30	Overweight or obese and 25(OH)D < 20 ng/mL	23	D <sub>3</sub> 100,000 IU once, then 4,000 IU/day (n = 33) versus placebo (n = 32)	16 weeks	↔ Peripheral Insulin sensitivity (clamp) ↔ AIR ↔ FG	Good
Cefalo (2018) (Italy)	40	Overweight (BMI >25 kg/m <sup>2</sup> ) and 25(OH)D < 30 ng/mL	14	D <sub>3</sub> 25,000 IU/week (n = 9) versus placebo (n = 9). All received hypocaloric diet.	12 weeks	↔ Insulin sensitivity (clamp)	Fair
Grubler (2021) (Switzerland)	70	Osteoarthritis	27	D <sub>3</sub> 2,000 IU/day (n = 33) versus 800 IU/day (n = 32). All received hypocaloric diet	2 years	↔ FG ↔ HOMA-IR	Fair
<b>Populations with type 2 diabetes</b>							
Sugden (2008) (UK)	64 (ND)	Stable type 2 diabetes and 25(OH)D < 20 ng/mL	15	D <sub>2</sub> 100,000 IU once (~1,785 IU/day) (n = 17) versus placebo (n = 17)	8 weeks	↔ HbA1c	Fair
Jorde (2009) (Norway)	56 (21–75)	Stable type 2 diabetes	24	D <sub>3</sub> 40,000 IU/week (~5,714 IU/day) (n = 16) versus placebo (n = 16)	26 weeks	↔ HbA1c ↔ FG	Fair
Parekh (2010) (India)	43 (35–50)	Type 2 diabetes	16	D <sub>3</sub> 300,000 IU intramuscular (n = 14) versus placebo (n = 14)	4 weeks	↔ FG ↔ HOMA-IR ↔ OGTT glucose ↔ OGTT insulin	Fair
Witham (2010) (United Kingdom)	65 (ND)	Type 2 diabetes and 25(OH)D < 40 ng/mL	18	D <sub>3</sub> 100,000 IU orally once (~892 IU/day) (n = 19) versus D <sub>3</sub> 200,000 IU orally once (~1,785 IU/day) (n = 20) versus placebo (n = 22)	16 weeks	↔ HbA1c (data ND) ↔ FG (data ND)	Fair
Soric (2012) (US)	54 (21–75)	Uncontrolled type 2 diabetes		D <sub>3</sub> 2,000 IU/day (n = 19) versus vitamin C 500 mg/day (n = 18)	12 weeks	↔ HbA1c ↓ HbA1c if baseline HbA1c > 9%	Poor
Elkassaby (2014) (Australia)	54 (48–58)	Type 2 diabetes	24	D <sub>3</sub> 10,000 IU/day (n = 26) for 2 weeks, then D <sub>3</sub> 6,000 IU/day versus placebo (n = 24)	26 weeks	↔ HbA1c ↔ FG ↔ PPG ↔ HOMA-IR	Fair

Continued



**TABLE 76.2** Randomized controlled trials of the effect of vitamin D (with or without calcium) on glucose tolerance in adults with normal glucose tolerance or established type 2 diabetes.—cont'd

First author year of publication (country)	Mean baseline age (range), y	Target population	Baseline 25(OH)D, ng/mL	Interventions (number of participants)	Study duration	Effect of vitamin D versus placebo/control	Study quality
Kampmann (2014) (Denmark)	60	Type 2 diabetes; and 25(OH)D < 20 ng/mL	13	D <sub>3</sub> 11,200 IU/day for 2 weeks, then D <sub>3</sub> 5,600 IU/day for 10 weeks (n = 8) versus placebo (n = 7)	12 weeks	↔ HbA1c ↔ Insulin sensitivity	Fair
Al-Sofiani (2015) (Saudi Arabia)	55 (21–75)	Type 2 diabetes and 25(OH)D < 20 ng/mL	13	D <sub>3</sub> 5,000 IU/day (n = 10) versus placebo (n = 10)	12 weeks	↔ HbA1c ↑ HOMA%B ↔ HOMA-IR	Poor
Krul-Poel (2015) (Netherlands)	67 ± 8	Type 2 diabetes	24	D <sub>3</sub> 50,000 IU/month (~1,666 IU/day) (n = 129) versus placebo (n = 132)	24 weeks	↔ HbA1c ↔ FG ↔ HOMA-IR and HOMA-B	Fair
Anayanwu (2016) (Nigeria)	35–65	Type 2 diabetes, non-insulin requiring with poor glycemic control and “vitamin D deficiency”	NA	D <sub>3</sub> 3,000 IU/day (n = 21) versus placebo (n = 21)	12 weeks	↓ HbA1c ↓ FG	Poor
Gulseth (2017)	56	Type 2 diabetes and 25(OH)D < 20 ng/mL	15	D <sub>3</sub> 400,000 IU once plus 200,000 IU once if needed to keep 25(OH)D > 40 ng/mL (n = 33) versus placebo (n = 29)	6 months	↔ HbA1c ↔ FG ↔ Fasting insulin/C-peptide ↔ AIR ↔ Glucose infusion rate	Fair
Upreti (2018) (India)	49	Type 2 diabetes and hypovitaminosis D	25	D 60,000 IU/week × 6, then monthly (n = 34) versus placebo (n = 37)		↓ HbA1c ↓ FG ↓ 2 hG	Poor
Khan (2018) (India)	40–70	Type 2 diabetes on metformin and 25(OH)D < 20 ng/mL	13	D <sub>3</sub> 50,000 IU/week (n = 70) versus placebo (n = 70)	12 weeks	↓ HbA1c [ <i>P</i> < .01]	Poor
Angellotti (2019) (US)	60	Stable type 2 diabetes (lifestyle or metformin)	27	D <sub>3</sub> 4,000 IU/day (n = 66) versus placebo (n = 61)	48 weeks	↔ Insulin secretion rate ↔ HbA1c ↔ FG ↔ 2 hG	Good

Lemieux (2019) (Canada)	40–70	High risk for type 2 diabetes or newly diagnosed type 2 diabetes on metformin and 25(OH)D < 20 ng/mL	20	D <sub>3</sub> 5,000 IU/day to reach 25(OH)D > 30 ng/mL (n = 48) versus placebo (n = 48)	6 months	↑ Peripheral insulin sensitivity (clamp) ↑ DI ↔ HbA1c ↔ FG ↔ 2 hG	Good
El Hajj (2020) (Lebanon)	66	Type 2 diabetes and 25(OH)D < 20 ng/mL	14	D <sub>3</sub> 10,000 IU 3 times a week (n = 48) versus placebo (n = 49)	6 months	↔ HbA1c ↔ FG	Fair
Cojic (2021) (Serbia)	50	Stable type 2 diabetes on metformin and 25(OH)D < 20 ng/mL	20 ng/mL	D <sub>3</sub> 50,000 IU/week for 3 months, then 14,000 IU/week for 3 months (n = 65) versus placebo (n = 65)	6 months	↓ HbA1c ↓ FG	Poor

↑, statistically significant increase (improvement vs. comparison group); ↓, statistically significant decrease (improvement vs. comparison group); ↔, no statistically significant change; 2 hG, glucose 2 h after a 75 g oral glucose load; 25(OH)D, plasma or serum 25-hydroxyvitamin D; AIR, acute insulin release; D<sub>2</sub>, ergocalciferol; D<sub>3</sub>, cholecalciferol; DI, disposition index (an estimate of pancreatic beta-cell function); FG, fasting glucose; HbA1c, glycated hemoglobin A1c; HOMA%B, homeostatic model assessment for insulin secretion; HOMA-IR, homeostatic model assessment for insulin resistance; HR, hazard ratio; IFG, impaired fasting glucose; IU, international units; ND, no data; NS, not significant; QUICKI, quantitative insulin-sensitivity check index. To convert 25(OH)D concentration from ng/mL to nmol/L multiply by 2.459; to convert FG from mg/dL to mmol/L, multiply by 0.0555.

advanced). In these trials, study duration varied from 2 months to 7 years and doses ranged from 400 IU/day to 88,865 IU/weekly. Studies that tested yogurt fortified with vitamin D are excluded from the discussion due to potential confounding effects of yogurt [204,205]. Due to several study limitations (e.g., small sample size [as small as 15 participants], short duration, open-label), the quality of most trials that have examined the effects of vitamin D on outcomes related to type 2 diabetes is rated as poor or fair.

Among trials of healthy people with normal glucose tolerance at baseline, vitamin D had no appreciable effect on glycemic measures [206–219] or incident diabetes [208,220]. All trials in people with normal glucose tolerance were not powered for glycemic outcomes. The two largest trials among people with normal glucose tolerance used doses of vitamin D (400 IU/day [208] and 800 IU/day [220]) that are considered inadequate for changing the pathophysiology of type 2 diabetes. Overall, vitamin D appears to have no effect among people with normal glucose tolerance, which is not surprising because it is very difficult to demonstrate statistically or clinically significant improvements in glycemic variables (e.g., fasting glucose, Hemoglobin A1c)—that are within the normal range at baseline.

Among trials in participants with normal glucose tolerance that reported insulin resistance, there was no statistically significant differences between vitamin D and placebo [207–209,212–216,218,219,221]. In contrast, the study by Von Hurst et al. randomized nondiabetic South Asian women that had blood 25(OH)D < 20 mg/dL (mean 8 ng/mL) to 4000 IU/day of vitamin D<sub>3</sub> (n = 42) or placebo (n = 39) for 6 months, and there was a significant improvement in insulin resistance, assessed by HOMA-IR, with vitamin D compared with placebo, which was more evident when endpoint serum 25(OH)D reached 32 mg/dL. In another trial among healthy men with blood 25(OH)D < 20 ng/mL (mean 16 ng/mL), vitamin D at 100,000 IU twice a month for 1 year maintained HOMA-IR compared with rising values in the control group [217]. Overall, vitamin D appears to have little or no effect on insulin resistance among people with normal glucose tolerance, especially if blood 25(OH)D is higher than 20 ng/mL, which is not surprising because people with normal glucose generally do not have insulin resistance to begin with.

There are at least 16 trials where participants with established type 2 diabetes were randomized to ergocalciferol [222] or cholecalciferol [223,224] [225,226,227,228,229,230,231,232,233,234,235,236,237,238] versus placebo or vitamin C [226] (Table 76.2). Among these trials, there is significant heterogeneity in how vitamin D was administered, duration of follow-up, and how outcomes were ascertained. Seven trials gave vitamin D daily

[226–229,231,235,236]. The remaining trials gave vitamin D as high (40,000 to 400,000 IU), nondaily doses whose benefit has been questioned [239–241]. It is now believed that to optimize bodily function, including in relation to insulin secretion and sensitivity, vitamin D should be available on a daily basis to ensure adequate stable circulating concentrations of the parent molecule [241].

In most trials among people with established type 2 diabetes, there was no change in measures of glycemia (fasting glucose, hemoglobin A1c or 2-hour glucose after a 75-gram glucose challenge) with vitamin D after a follow-up period of 4 weeks to 1 year. Four trials reported favorable changes in hemoglobin A1c or fasting glucose in the group that was randomized to vitamin D [231,233,238]. What these trials have in common is that they recruited patients with stable, noninsulin requiring type 2 diabetes and low baseline blood 25(OH)D level (generally less than 20 ng/mL). Overall, results suggest that vitamin D may not have an appreciable effect on glycemia in people with established type 2 diabetes; however, firm conclusions from these trials cannot be drawn as the studies were underpowered and there was high heterogeneity in populations studies. Most importantly, given the multiple factors that can influence glycemia during a trial that can be difficult to control, an adequately powered (very large) study that carefully monitors and adjusts for potential confounders between active and placebo arms (e.g., concurrent hypoglycemic medications, lifestyle habits) is needed to test the effect of vitamin D among patients with type 2 diabetes. Such trial is unlikely to be conducted. The Thiazolidinedione Intervention with vitamin D Evaluation (TIDE) study was a 3 × 2 factorial design trial aimed to assess the effects of thiazolidinediones (rosiglitazone and pioglitazone) and 1000 IU/day of vitamin D<sub>3</sub> among 16,000 patients with type 2 diabetes in 33 countries [242]. The trial was expected to provide important data on the effect of vitamin D in people with type 2 diabetes; however, it was stopped prematurely because of regulatory concerns regarding the thiazolidinediones.

Two trials have reported the effect of combined vitamin D<sub>3</sub> and calcium supplementation versus placebo on glycemic measures and diabetes risk, both *post hoc*. In the WHI trial, vitamin D<sub>3</sub> (400 IU/day) and calcium supplementation (1000 mg/day) did not reduce the risk of developing self-reported diabetes over a 7-year period, and there was also no significant effect on fasting glucose or simple indices of insulin resistance [208]. The WHI trial used a small dose of vitamin D and allowed participants to take vitamin D supplements on their own during the trial. Although the effect of supplementation on blood 25(OH)D concentration was not reported, based on dose and adherence, it has been estimated it to be only 2 ng/mL [243], which is an increment

unlikely to have any effect. In the trial by Pittas et al. in the subgroup of participants with impaired fasting glucose at baseline, combined vitamin D<sub>3</sub> (700 IU/day) and calcium carbonate (500 mg/day) attenuated the increase in fasting glycemia that occurs over time in this population [207]. There was no effect on glycemia among participants with normal glucose tolerance at baseline, suggesting that vitamin D may benefit only individuals with prediabetes. In another trial by Pittas et al. among people with prediabetes [50], vitamin D and calcium were given in a 2 × 2 factorial design. When examining the group that was randomized to receive both vitamin D and calcium, there was a significant improvement in both hemoglobin A1c and fasting glucose, suggesting that combination of vitamin D and calcium may be important in the modulation of the pathophysiology of type 2 diabetes. However, the sample size was very small, and firm conclusions cannot be drawn on the importance of combining vitamin D with calcium to optimize diabetes outcomes.

In summary, vitamin D supplementation has not been shown to be of benefit in studies that included patients with normal glucose tolerance or established type 2 diabetes, but there appears to be a role in people at risk for diabetes (prediabetes). synthesis of the available evidence from trials on the effect of vitamin D on new-onset diabetes and regression to normal glucose regulation among people with prediabetes is presented in the next section.

## 6. Vitamin D for prevention of type 2 diabetes in people with prediabetes

### 6.1 Vitamin D and new-onset diabetes. Results from individual clinical trials

Thirteen trials published between 2008 and 2022 have reported on the effect of vitamin D on new-onset diabetes [4–6,208,220,244–251] (Table 76.3). Two large trials (WHI and RECORD) were designed and conducted for nondiabetes outcomes and reported data on new-onset diabetes in ancillary, post hoc analyses [208,220]. These two trials enrolled patients at average risk for diabetes, the intervention was low-dose vitamin D<sub>3</sub> (400 and 800 IU daily) coadministered with calcium in a 2 × 2 factorial design, and diabetes was ascertained by participant self-report based on a diagnosis made outside of the study in routine clinical practice. The remaining 11 trials that have reported data on the effect of vitamin D supplementation and incident diabetes recruited adults with prediabetes [4–6,244–251]. Seven of these trials have major limitations, including small sample size (90–205 participants) [244–251], not designed or powered for new-onset diabetes as the primary outcome [244–251], short duration

(=<1 year) [244,246–248,251], or open-label study design without placebo [245,247,249–251]. In most of these trials, including the two largest ones [208,220], adherence with supplementation was suboptimal, which further limit drawing any conclusions. For example, in a post hoc analysis of the RECORD study, a community-based effectiveness trial designed for bone outcomes, supplementation with 800 IU/day of vitamin D<sub>3</sub> did not change risk of self-reported diabetes; however, among participants who were highly adherent with supplementation, there was a notable trend toward reduction in diabetes risk with vitamin D<sub>3</sub> (odds ratio 0.68; 95% confidence interval 0.40, 1.16) [220], which highlights the difference of efficacy (where adherence is monitored and encouraged) versus effectiveness community-based trials when evaluating evidence. Overall, results from the two large trials (WHI, RECORD) [208,220] and these eight smaller trials [244–251] are not informative in our understanding of the role of vitamin D supplementation for prevention of type 2 diabetes in the clinical setting.

There have been three large, double-blind, placebo-controlled, randomized clinical trials designed and conducted specifically to test the hypothesis that vitamin D lowers the risk of developing diabetes among persons with prediabetes [4–6,252,253] (Table 76.4).

#### 6.1.1 The Tromsø study

The Tromsø study was a single-site trial that took place from March 2008 through March 2015 in Norway [5]. The study randomly assigned 511 adults (mean age 62 years, mean BMI 30 kg/m<sup>2</sup>) who met at least one of two glycemic criteria for prediabetes (fasting glucose 108–125 mg/dL; glucose 2 h post 75-gram oral glucose load 140–199 mg/dL) and had no diabetes (fasting glucose <126 mg/dL and hemoglobin A1c <6.5%) to treatment with 20,000 IU of vitamin D<sub>3</sub> weekly (equivalent to ~2900 IU per day) or placebo. Blood 25(OH)D level was not an eligibility criterion. The primary outcome was time to new-onset diabetes based on annual glycemic testing through fasting glucose, hemoglobin A1c, and 2-hour glucose. The study had 80% power to detect a risk reduction in new-onset diabetes of 30% with vitamin D compared with placebo. Mean baseline blood 25(OH)D level was 24 ng/mL, and 68% of participants had a blood 25(OH)D level ≥20 ng/mL (Table 76.4). During follow-up, mean blood 25(OH)D level in the vitamin D group rose to 44 ng/mL compared with 26 ng/mL in the placebo group. After a median follow-up of 2.5 years, 103 participants developed diabetes in the vitamin D group versus 112 in the placebo group (11.8 events vs. 13.1 events per 100 person-years, respectively). In the intention-to-treat analysis, the risk of diabetes was 10% lower in the vitamin D group, but the result was not statistically significant (hazard ratio for vitamin D 0.90; 95% confidence interval



**TABLE 76.3** Trials that have reported on the effect of vitamin D and new-onset diabetes or regression to normal glucose regulation.

First author year of publication (country)	Target population (n = number of participants)	Baseline 25(OH)D concentration, ng/mL	Interventions (number of participants)	Study duration, years	Study quality (reasoning)	Designed for glycemic outcomes, (Yes/ No)	Primary outcome(s)	Other glycemic outcomes?	Was the trial designed, conducted for the primary prevention of type 2 diabetes, i.e., was new-onset diabetes the primary outcome?
<i>Populations at average risk for type 2 diabetes – not designed for glycemic outcomes or for primary prevention of type 2 diabetes</i>									
De Boer (2008) (US)	Healthy, postmenopausal women (n = 33,951)	Not available	D <sub>3</sub> 400 IU daily plus calcium carbonate 1,000 mg daily (n = 16,999) versus placebo (n = 16,952)	Median of 7 years	Fair (post hoc analysis, self- report of new- onset diabetes, single gender)	No	- Fracture reduction	- New-onset diabetes	No
Avenell (2009) (UK)	Healthy adults ≥70 years and history of fracture (n = 5,292)	Not available	D <sub>3</sub> 800 IU daily (n = 2,649) versus placebo (n = 2,643) (2 × 2 factorial design with calcium carbonate 1,000 mg daily)	Range of 2–5 years	Fair (post hoc analysis, self- report of new- onset diabetes)	No	- Fracture reduction	- New-onset diabetes	No
<i>Populations at risk for type 2 diabetes (prediabetes) – not designed for primary prevention of type 2 diabetes</i>									
Davidson (2013) (US)	Prediabetes by FG or 2 hG, and 25(OH) D < 30 ng/mL (n = 109)	22	Treat-to-target 25(OH)D 65 –90 ng/mL, mean D <sub>3</sub> 88,865 IU weekly (~12,695 IU daily) (n = 56) versus placebo (n = 53)	Up to 1 year	Moderate (small sample size)	Yes	- OGTT-based insulin sensitivity and secretion	- New-onset diabetes - NGR	No
Dutta (2014) (Eastern India)	Prediabetes by FG or 2 hG, and 25(OH) D < 30 ng/mL (n = 125)	17	D <sub>3</sub> 60,000 IU weekly (~8,571 IU daily) for 8 weeks, then 60,000 IU monthly (~3,000 IU daily) (n = 68) versus no treatment (n = 57) (open label). All received calcium 500 mg daily	Mean of 2.3 years	Poor (small sample size, open label, unclear methodology section)	Yes	- New-onset diabetes - NGR	- FG - 2 hG	No

Barengolts (2015) (US)	Black men with prediabetes (84%) or diabetes (16%) based on FG or HbA1c, and 25(OH)D 5–29 ng/mL, and prevalent medical problems (n = 205)	14	D <sub>2</sub> 50,000 IU weekly (~7,143 IU daily) adjusted to achieve 25(OH)D 40–100 ng/mL (n = 103) versus placebo (n = 102). All received D <sub>3</sub> 400 IU daily	Up to 1 year	Fair (small sample size, single race and gender; both prediabetes and diabetes populations)	Yes	- OGTT-based oral glucose insulin sensitivity	- New-onset diabetes - NGR - OGTT-based insulin secretion - HbA1c	No
Kuchay (2015) (North India)	Prediabetes by FG, 2 hG, or HbA1c (n = 137)	19	D <sub>3</sub> 60,000 IU weekly (~2,000 IU daily) for 4 weeks, then monthly (n = 69) versus no treatment (n = 68) (open label)	Up to 1 year	Poor (small sample size, open label)	Yes	- FG - 2 hG - HbA1c	- New-onset diabetes	No
Niroomand (2019) (Iran)	Prediabetes based on FG or 2 hG, and 25(OH)D < 30 ng/mL (n = 162)	13	D <sub>3</sub> 50,000 IU weekly (~7,143 IU daily) for 3 months, then monthly (n = 81) versus placebo (n = 81)	Up to 6 months	Poor (small sample size, completers only analysis [50% of randomized])	Yes	- Insulin sensitivity	- New-onset diabetes - NGR - FG - 2 hG	No
Bhatt (2020) (India)	Prediabetes based on FG or 2 hG, and 25(OH)D < 20 ng/mL (n = 121)	12	D <sub>3</sub> 60,000 IU weekly (~3,571 IU daily) for 8 weeks and repeated as needed to "avoid deficiency," then 200 IU daily (n = 61) versus placebo (n = 60) (open label). All received calcium carbonate daily	18 months	Poor (small sample size, open label)	Yes	- FG - 2 hG - HbA1c - New-onset diabetes - NGR		No
Misra (2021) (Northern India)	Prediabetes based on FG or 2 hPG and 25(OH)D < 30 ng/mL	23	D <sub>3</sub> 60,000 IU weekly (~8,571 IU daily) for 8 weeks, repeated if needed to reach 25(OH)D > 30 ng/mL (n = 67) versus placebo (n = 65) (open label)	Up to 2 years	Poor (small sample size, open label)	Yes	- New-onset diabetes	- NGR - FG - 2 hG - HbA1c	No

Continued

**TABLE 76.3** Trials that have reported on the effect of vitamin D and new-onset diabetes or regression to normal glucose regulation.—cont'd

First author year of publication (country)	Target population (n = number of participants)	Baseline 25(OH)D concentration, ng/mL	Interventions (number of participants)	Study duration, years	Study quality (reasoning)	Designed for glycemic outcomes, (Yes/ No)	Primary outcome(s)	Other glycemic outcomes?	Was the trial designed, conducted for the primary prevention of type 2 diabetes, i.e., was new-onset diabetes the primary outcome?
Zaromytidou (2022) (Greece)	Prediabetes based on FG or 2 hG or HbA1c (n = 90)	20	D <sub>3</sub> 25,000 IU weekly (~3,571 IU daily) (n = 45) versus no treatment (n = 45) (open label)	Up to 1 year	Poor (small sample size, open label)	Yes	- FG - 2 hG - HbA1c	- New-onset diabetes - NGR	No
<i>Populations at risk for type 2 diabetes (prediabetes) – designed for primary prevention of type 2 diabetes</i>									
Jorde (2016) (Norway)	Prediabetes by FG or 2 hG (n = 511)	24	D <sub>3</sub> 20,000 IU weekly (~2,857 IU daily) (n = 256) versus placebo (n = 255)	Up to 5 years	Good	Yes	- New-onset diabetes	- NGR - FG - 2 hG - HbA1c	Yes
Pittas (2019) (US)	Prediabetes by FG or 2 hG or HbA1c (n = 2,423)	28	D <sub>3</sub> 4,000 IU daily (n = 1,211) versus placebo (n = 1,212)	Median 2.5 years (event- driven)	Good	Yes	- New-onset diabetes	- NGR - FG - 2 hG - HbA1c	Yes
Kawahara (2022) (Japan)	Prediabetes by 2 hG (n = 1,256)	21	Eldecalcitol 0.75 µg daily (n = 630) versus placebo (n = 626)	Up to 3 years	Good	Yes	- New-onset diabetes	- NGR - FG - 2 hG - HbA1c	Yes

2 hG, glucose 2 h after a 75 g oral glucose load; 25(OH)D, plasma or serum 25-hydroxyvitamin D; D<sub>2</sub>, ergocalciferol; D<sub>3</sub>, cholecalciferol; FG, fasting glucose; HbA1c, glycated hemoglobin A1c; IU, international units; NGR, normal glucose regulation; OGTT, oral glucose tolerance test. To convert 25(OH)D concentration from ng/mL to nmol/L multiply by 2.459; to convert FPG from mg/dL to mmol/L, multiply by 0.0555.

**TABLE 76.4** Key characteristics of the randomized, double-blind, placebo-controlled clinical trials with vitamin D for prevention of diabetes among adults at risk for type 2 diabetes (prediabetes).

Study name	Tromsø study	D2d study	DPVD study
First author, year of publication	Jorde et al. (2016)	Pittas et al. (2019)	Kawahara et al. (2022)
Country (number of sites) <sup>a</sup>	Norway (1 site)	United States (22 sites)	Japan (3 sites)
Year of trial completion	2015	2018	2019
Randomized participants (vitamin D:placebo), no.	511 (256:255)	2423 (1211:1212)	1256 (630:626)
Prediabetes glycemic criteria for eligibility	IFG (FG 108–125 mg/dL) and/or IGT (2 hG 140–199 mg/dL) and no criterion in the diabetes category	Two or three glycemic criteria (IFG (FG 100–125 mg/dL), IGT (2 hG 140–199 mg/dL), iA1c (HbA1c 5.7%–6.4%)) and no criterion in the diabetes category	IGT (2 hG 140–199 mg/dL) and no criterion in the diabetes category
Baseline blood 25(OH)D, ng/mL	24	28	21
Percent of participants with blood 25(OH)D above 20 ng/mL	62	80	54
Intervention	Cholecalciferol (vitamin D <sub>3</sub> ), 20,000 IU weekly (equivalent to 2,857 IU daily)	Cholecalciferol (vitamin D <sub>3</sub> ), 4,000 IU daily	Eldecalcitol, 0.75 µg daily
Comparator	Placebo daily	Placebo daily	Placebo daily
Vitamin D amount from supplements allowed outside of the study	≤400 IU/day	≤1000 IU/day	No vitamin D from supplements was allowed
Definition of primary outcome, new-onset diabetes	Any glycemic-positive criteria: FG ≥ 126 mg/dL, 2 hG ≥ 200 mg/dL, HbA1c ≥ 6.5%. A positive HbA1c required confirmation	Two or three glycemic-positive criteria: FG ≥ 126 mg/dL, 2 hG ≥ 200 mg/dL, HbA1c ≥ 6.5%, or 1 criterion positive with confirmation	HbA1c ≥ 6.5% and either: FG ≥ 126 mg/dL, 2 hG ≥ 200 mg/dL, or casual glucose ≥ 200 mg/dL
Expected incidence of diabetes in the placebo group, per 100 person-years	10.0	10.5	8.4
Expected relative risk reduction in diabetes, vitamin D versus placebo, %	30	25	36
Median (interquartile range) (range) follow-up, years <sup>b</sup>	4.0 (1.5–5.0) (0.2–5.2)	2.5 (1.9–3.5) (0–4.7) (event-driven)	2.9 (2.8–3.0) (0.1–3.0)
Hazard ratio (95% confidence interval) for new-onset diabetes, vitamin D versus placebo	0.90 (0.69–1.18)	0.88 (0.75–1.04)	0.87 (0.67–1.17)

<sup>a</sup>Tromsø had 1 site in Norway (University Hospital of North Norway at 70° North latitude); D2d had 22 sites (22 different locations) in the United States (12 sites were above 37° North latitude and 10 sites were below 37° North latitude); DPVD had 3 sites at 2 locations in Japan (Fukuoka location at 34° North latitude and Kanagawa location at 35° North latitude).

<sup>b</sup>The Tromsø study was designed to follow participants up to 5 years or until the diagnosis of diabetes. The D2d study was designed as an event-driven trial and follow-up, as expected, varied among participants. The DPVD study was designed to follow participants up to 3 years or until the diagnosis of diabetes.

2 hG, glucose 2 h after a 75-gram oral glucose load; FG, fasting glucose; HbA1c, hemoglobin A1c; iA1c, impaired hemoglobin A1c; IFG, impaired fasting glucose; IGT, impaired glucose tolerance.

0.69–1.18) [5]. The authors reported that among participants with a rise in blood 25(OH)D from less than 20 ng/mL to more than 32 ng/mL, there was a larger risk reduction (21%), but the change was also not statistically significant due to the small number of participants in this subgroup (hazard ratio for vitamin D 0.79; 95% confidence interval 0.46–1.37).

### 6.1.2 The vitamin D and type 2 diabetes study

The D2d study was the largest vitamin D and diabetes prevention trial with 22 sites in the United States and took place from October 2013 through December 2018 (Fig. 76.5) [4,253]. The study, which was supported by the National Institutes of Health, randomly assigned 2423 adults (mean age 60 years, mean BMI 32.1 kg/m<sup>2</sup>)



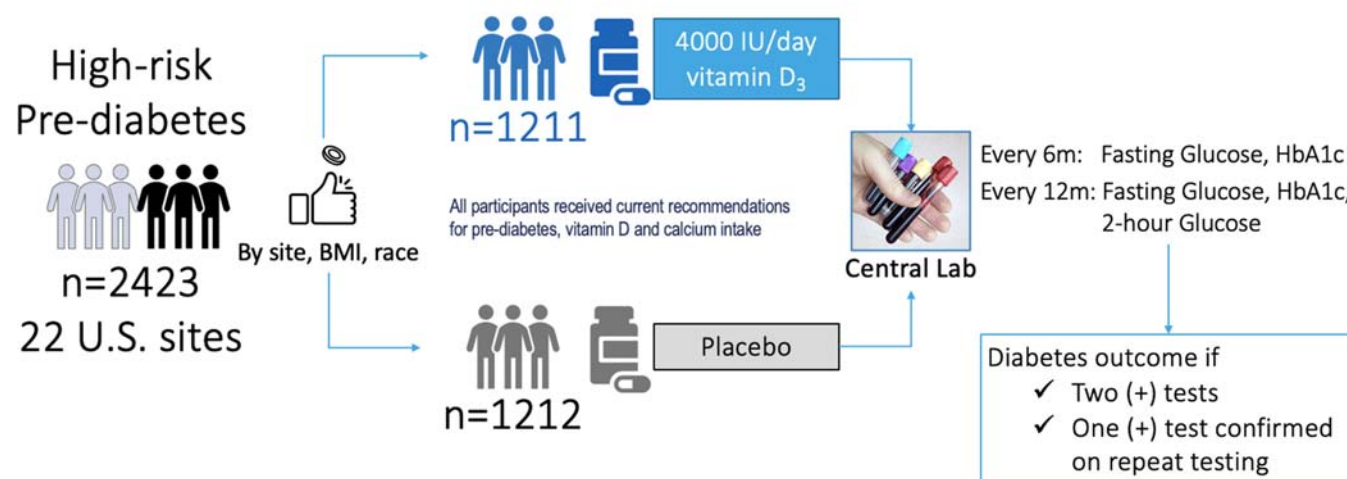


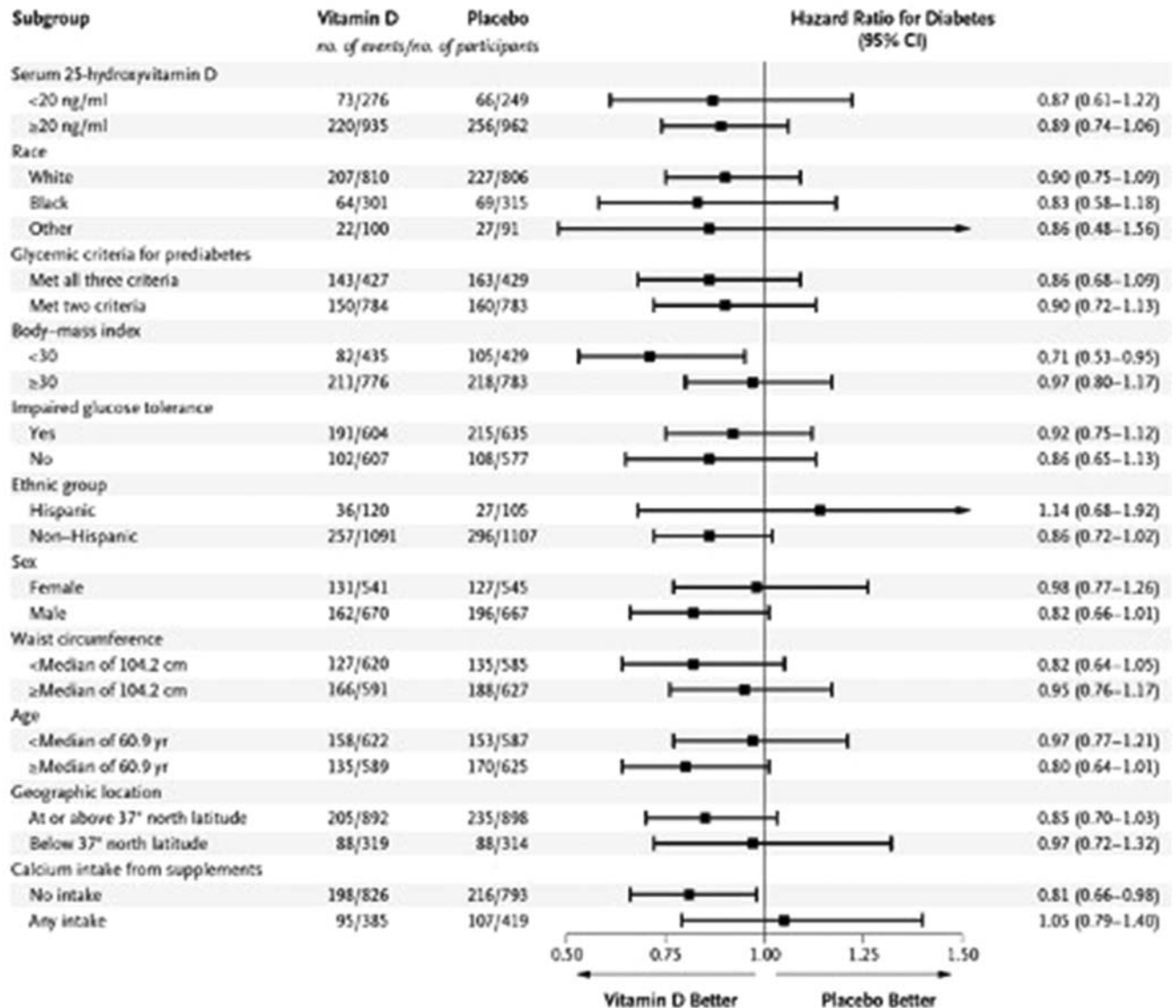
FIGURE 76.5 Overview of the design of the vitamin D and type 2 diabetes (D2d) study.

who met at least two of three glycemic criteria for pre-diabetes (fasting glucose 100–125 mg/dL; glucose 2 h post 75-gram oral glucose load 140–199 mg/dL; hemoglobin A1c 5.7%–6.4%) to treatment with 4000 IU of vitamin D<sub>3</sub> daily or placebo. The primary outcome was time to new-onset diabetes based on annual glycemic testing through fasting glucose, hemoglobin A1c, and 2-hour glucose, and semiannual testing with fasting glucose and hemoglobin A1c. The D2d study was designed as an event-driven trial, and blood 25(OH)D level was not an eligibility criterion. The study had 90% power to detect a risk reduction in new-onset diabetes of 25% with vitamin D compared with placebo. Mean baseline blood 25(OH)D level was 28.0 ng/mL, and 78% of participants had a 25(OH)D level  $\geq 20$  ng/mL (Table 76.4). At baseline, 84% of participants met both fasting glucose and hemoglobin A1c criteria for prediabetes, which are the most commonly criteria for prediabetes used in clinical practice. During follow-up, mean blood 25(OH)D level in the vitamin D group rose to 54 ng/mL compared with 29 ng/mL in the placebo group. After a median follow-up of 2.5 years, 293 participants in the vitamin D group developed diabetes versus 323 in the placebo group (9.39 events vs. 10.66 events per 100 person-years, respectively). In the intention-to-treat analysis, the risk of diabetes was 12% lower in the vitamin D group, but the result was not statistically significant (hazard ratio for vitamin D 0.88; 95% confidence interval 0.75–1.04) [4]. The results of the subgroup analyses were consistent with the findings of the main analysis, i.e., favoring vitamin D but without statistically significant heterogeneity among subgroup (Fig. 76.6), except among participants with a baseline 25(OH)D level  $< 12$  ng/mL (103 participants), where vitamin D reduced the risk of developing diabetes by

62% (hazard ratio for vitamin D 0.38; 95% confidence interval 0.18–0.80) (Fig. 76.7).

### 6.1.3 Diabetes prevention with active vitamin D study

The DPVD study was a three-site trial that took place from June 2013 through August 2019 in Japan [6,252]. The study randomly assigned 1256 adults (mean age 61 years, mean BMI 24 kg/m<sup>2</sup>) who met the impaired glucose tolerance criterion for prediabetes (glucose 2 h post 75-gram oral glucose load 140–199 mg/dL) and had no diabetes (fasting glucose  $< 126$  mg/dL and hemoglobin A1c  $< 6.5\%$ ) to treatment with 0.75 mg of eldcalcitol (an analog of calcitriol, the active form of vitamin D<sub>3</sub>) daily or placebo. Blood 25(OH)D level was not an eligibility criterion. The primary outcome was time to new-onset diabetes based on annual glycemic testing through fasting glucose, hemoglobin A1c, and 2-hour glucose and quarterly glycemic testing with fasting glucose and hemoglobin A1c. The study had 80% power to detect a risk reduction in new-onset diabetes of 36% with vitamin D compared with placebo. Mean baseline blood 25(OH)D level was 21 ng/mL, and 56% of participants had a 25(OH)D level  $\geq 20$  ng/mL (Table 76.4). During follow-up, mean blood 25(OH)D level did not change in the vitamin D or placebo groups, as expected because eldcalcitol is the active form of vitamin D<sub>3</sub>. After a median follow-up of 2.9 years, 79 participants developed diabetes in the vitamin D group versus 89 in the placebo group (12.5 vs. 14.2%, respectively). In the intention-to-treat analysis, the risk of diabetes was 13% lower in the vitamin D group, but the result was not statistically significant (hazard ratio for vitamin D 0.87; 95% confidence interval 0.67–1.17).



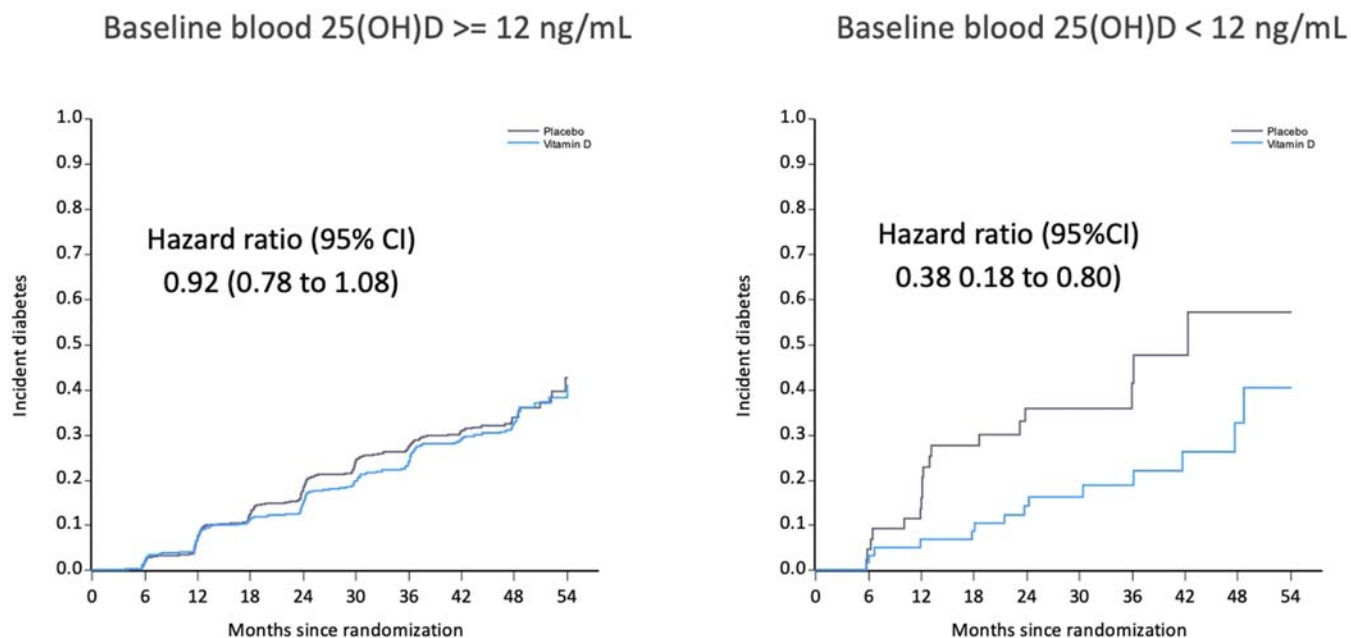
**FIGURE 76.6** Hazard ratios for new-onset diabetes in the vitamin D and type 2 diabetes (D2d) study in pre-defined subgroups of participants defined by baseline characteristics. From Figure 3 in Ref. [4].

[6]. After adjustment for confounding factors by multi-variable fractional polynomial Cox regression analysis, there was a statistically significant reduction in the risk of developing diabetes of 31% in the eldecacitol group compared with the placebo group (hazard ratio for vitamin D 0.69, 95% confidence interval 0.51–0.95). Eldecacitol had a significant effect on diabetes risk among those with insufficient insulin secretion.

#### 6.1.4 Other large trials

Except for the Tromsø, DPVD, and D2d studies, there are no other vitamin D and diabetes prevention trials that have been specifically designed and conducted to

test the effect of vitamin D on reducing risk of developing type 2 diabetes. There are several large trials testing the effect of vitamin D supplementation on non-diabetes outcomes (e.g., The VITamin D and Omega-3 Trial [VITAL] [254], D-Health [255], the Finnish Vitamin D Trial (FIND, NCT01463813) [256] in populations at average risk for diabetes, and these trials may report results on diabetes risk, based on ancillary secondary analyses. However, results will have the same limitations as all other trials that are not designed for diabetes prevention (e.g., enrollment of populations at average risk for diabetes, inadequate vitamin D dose, and insufficiently defined and ascertained diabetes outcome).



**FIGURE 76.7** Hazard ratio for new-onset diabetes in the vitamin D and type 2 diabetes (D2d) study among participants with prediabetes and baseline blood 25(OH)D  $\geq 12$  ng/mL (left panel, N = 2319) or baseline blood 25(OH)D  $< 12$  ng/mL (right panel, N = 103). Nominal *P*-value for the interaction term = .023.

## 6.2 Vitamin D and new-onset diabetes. Results from aggregate (study-level) data metaanalyses

Two metaanalyses have combined aggregate (study-level) data from vitamin D supplementation trials that have reported on new-onset diabetes [2,257].

Zhang et al. synthesized results from eight trials (total of 4896 participants, sample sizes ranging from 117 to 2423; duration of follow-up 6 months to 5 years) in persons with prediabetes. Three of the included trials had a low risk for bias (Tromsø, D2d, and DPVD) [4–6], and the rest had either unclear or high risk of bias. The authors reported a benefit of vitamin D for new-onset diabetes (risk ratio 0.89; 95% confidence interval 0.80–0.99) [257]. There was no publication bias. In subgroup analyses, the benefit was prominent when combining aggregate results from trials that had a mean baseline nonobese BMI ( $<30$  kg/m<sup>2</sup>) (risk ratio 0.73; 95% confidence interval 0.75–0.92) versus trials that had a mean baseline obese BMI (risk ratio 0.95; 95% confidence interval 0.84–1.08). The authors did not find any interaction according to mean baseline 25(OH)D, achieved 25(OH)D in vitamin group, dose of vitamin D, length of follow-up, intervention, or latitude. Overall, the authors concluded that in people with prediabetes, vitamin D reduces the risk of developing type 2 diabetes.

Barbarawi et al. synthesized results from nine trials (total of 43,559 participants; sample sizes ranging from 1109 to 33,951; duration of follow-up 1–7 years) that reported the effect of vitamin D for at least 1 year on

new-onset diabetes [2]. Two trials (WHI, RECORD) (total of 39,243 participants) were designed and conducted for nondiabetes outcomes (fracture reduction) in persons of average diabetes risk who were randomized to low-dose vitamin D ( $<1000$  IU per day) and the trials reported data on new-onset diabetes in post hoc analyses [208,220]; seven trials (total of 4316 participants) were in persons with prediabetes randomized to moderate–high doses of vitamin D ( $\geq 1000$  IU per day). The authors reported a benefit of vitamin D for new-onset diabetes when combined data from the trials among persons with prediabetes who also received moderate–high doses of vitamin D (risk ratio 0.88; 95% confidence interval 0.79–0.99). In contrast, when combining data from the trials that tested lower doses, which were also conducted in the general average-risk-for-diabetes population, there was no risk reduction with vitamin D (risk ratio 1.02; 95% confidence interval 0.94–1.10; *P* for interaction = 0.04). There was no publication bias. Like the metaanalysis by Zhang et al. the authors reported that the benefit of vitamin D was seen when combining trials that had a mean baseline nonobese BMI ( $<30$  kg/m<sup>2</sup>) (risk ratio 0.68; 95% confidence interval 0.53–0.89), while there was no benefit among trials with a mean baseline BMI  $\geq 30$  kg/m<sup>2</sup>. Subgroup analysis according to baseline mean age, gender, formulation (daily vs. bolus dosing), and low mean baseline blood 25(OH)D level did not reveal any significant modifying effects. Overall, the authors concluded that in people with prediabetes, vitamin D at moderate or high doses ( $\geq 1000$  units per day) reduces the risk of developing type 2 diabetes.



Metaanalyses have increased in popularity, but even if the methodology is sound, the strength of a metaanalysis result depends on the quality of the included individual trials. Most importantly, for metaanalyses to draw appropriate conclusions, they must combine data from trials that have been specifically designed and conducted to answer the question at hand. Although the metaanalyses by Zhang et al. and Barbarawi et al. used data from clinical trials, there are many limitations. Trials that simply report on incident diabetes as a secondary outcome do not carry the same weight as trials that have been specifically designed and conducted to test the hypothesis that vitamin D supplementation reduces diabetes risk. For example, the WHI study was a “mega” trial that used a relatively small dose of vitamin D and was designed and conducted to test nondiabetes outcomes in a population at average risk for diabetes and assessed the diabetes outcome by participant’s self-report of taking pills or insulin for diabetes. Therefore, including trials such as the WHI in metaanalyses of vitamin D and diabetes prevention trials is methodologically inappropriate, and given their large size, results would dilute the overall result of the metaanalysis, leading to inappropriate conclusions. In fact, except for three trials (Tromsø, D2d, and DPVD) [4–6], all other trials that were in both these metaanalyses were also not designed for diabetes prevention.

Interestingly, both metaanalyses reported on the effects of vitamin D in subgroups by baseline BMI cutoffs; however, such a subgroup claim is highly problematic and may be erroneous (subject to ecological fallacy) because the authors generated analyses based on the average BMI of each trial cohort rather than the BMI of each participant [89]. For example, trials with mean baseline BMI  $<30$  kg/m<sup>2</sup> include participants with BMI  $\geq 30$  kg/m<sup>2</sup> in both vitamin D and placebo groups and using the mean cohort BMI to gain insight into whether vitamin D works differentially among people with different BMI is methodologically flawed. The same limitations apply to the subgroup analyses according to other variables at baseline (e.g., age, blood 25(OH)D levels). In the absence of individual participant data, most results from subgroups analyses in aggregate data metaanalyses are uninterpretable.

Due to similar limitations, results by the recent USPSTF report on the effect of vitamin D on diabetes risk in people with vitamin D deficiency are not informative [258]. The USPSTF report pooled data from five highly heterogeneous trials (varied interventions [vitamin D or vitamin plus calcium], study populations [prediabetes or average risk], and outcome assessments [in-study diagnosis, self-report, or as adverse event] and did not include the DPVD study, the second largest vitamin D and diabetes prevention trial.

### 6.3 Vitamin D and new-onset diabetes. Results from individual participant data metaanalysis

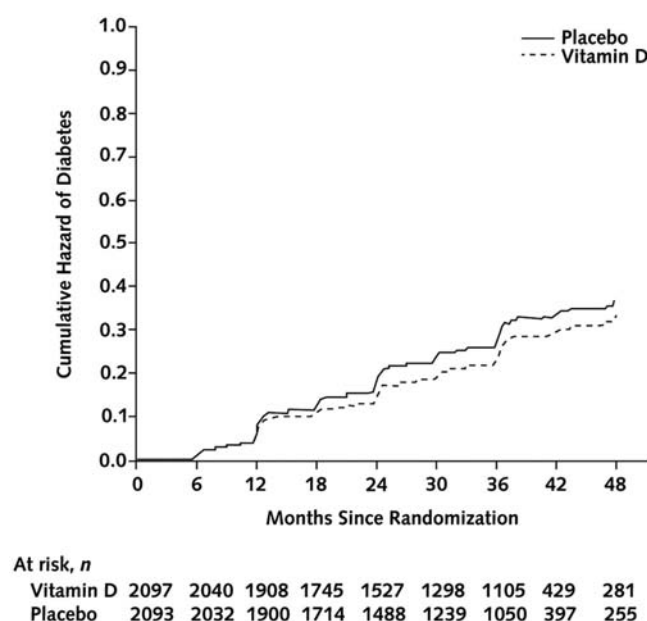
In contrast to an aggregate data metaanalysis, an individual participant data metaanalysis offers several advantages: increased statistical power to detect benefits and risks, higher precision of results (i.e., narrower 95% confidence interval), ability to conduct a detailed analysis including sensitivity analyses to test the robustness of results, define more precisely participant cohorts and standardize covariate definitions, and harmonize analyses to attenuate methodological heterogeneity; establish differences among important subgroups by baseline characteristics, which allows for identification of segments of the population most likely to benefit from supplementation [259–263].

Pittas et al. conducted a systematic review of the published literature and performed an individual participant data metaanalysis strictly of randomized, placebo-controlled trials of vitamin D—only among adults with prediabetes to assess whether vitamin D decreases the risk of new-onset diabetes and whether the effect differs across prespecified subgroups of participants [264].

Three trials met eligibility criteria: Tromsø (tested cholecalciferol), DPVD (tested eldecalcitol), and D2d (tested cholecalciferol) studies [4–6]. All 4190 participants from the three trials contributed data to the metaanalysis (N = 2097 were randomly assigned to receive vitamin D and N = 2093 to receive placebo). Mean age of the combined cohort with prediabetes was 61 years; 44% were women, 51% self-identified their race as White or European 33% as Asian, and 15% as Black. Mean BMI was 30 kg/m<sup>2</sup>, and mean serum 25(OH)D level was 25 ng/mL.

During a median follow-up of 3.0 years, new-onset diabetes occurred in 22.7% of participants in the vitamin D versus 25.0% in the control group (Fig. 76.8). The unadjusted hazard ratio for vitamin D was 0.88 (95% confidence interval 0.77–0.99) in the intention-to-treat analysis and 0.85 (95% confidence interval 0.75–0.97) in the “as treated” (efficacy) analysis. Adjusted hazard ratios were 0.85 (95% confidence interval 0.75–0.96) in the intention-to-treat and 0.83 (95% confidence interval 0.73–0.94) in the “as treated” analysis. The effect of vitamin D (cholecalciferol or eldecalcitol combined) did not differ in prespecified subgroups. The effect of vitamin D on new-onset diabetes did not differ by baseline age, BMI, gender, race, glycemic risk, or calcium intake from supplements. Among the 224 participants with baseline 25(OH)D level less than 12 ng/mL, the vitamin D reduced the risk of diabetes by 62% (hazard ratio 0.58 [95% CI 0.35–0.97]). When the authors examined data from the two trials that administered





**FIGURE 76.8** Incidence curves for new-onset diabetes among adults with prediabetes: intention to treat analysis of individual participant data from the three vitamin D and diabetes prevention trials (Tromsø, D2d, DPVD). From Figure 2 in Ref. [273].

cholecalciferol, which requires activation to 25(OH)D in the liver and elsewhere by CYP2R1 [4,5], the effect of cholecalciferol on new-onset diabetes was noted only in participants with below median BMI (hazard ratio 0.74 [95% confidence interval 0.60–0.90] for BMI <31 versus 1.01 [95% confidence interval 0.84–1.22] for BMI ≥31 kg/m<sup>2</sup>).

The key strength of this individual participant data metaanalysis lies in the high quality of the included clinical trials, all being double-blinded, randomized, placebo-controlled, and at low risk of bias. Most importantly and in contrast to other metaanalyses in this area that combined data from trials with high-risk for bias [2,257], the eligible trials in this metaanalysis were specifically designed and conducted for diabetes prevention, used modern definitions of prediabetes to define the at-risk-for-diabetes study populations, had adequate long-term follow-up, and ascertained the primary outcome of new-onset diabetes by prespecified glycemic criteria, as defined by the American Diabetes Association or the World Health Organization.

#### 6.4 Vitamin D and regression to normal glucose regulation. Results from individual clinical trials

Although diabetes prevention trials focus on delaying progression from prediabetes to diabetes, regression to normal glucose regulation is an important clinical outcome because euglycemia is associated with a lower

prevalence of microvascular disease compared with prediabetes, likely due to less long-term exposure to abnormal glucose levels and because even transient regression to normal glucose regulation confers lower risk of developing diabetes [264–266]. In addition to reducing progression from prediabetes to diabetes, vitamin D increases the likelihood of regression to normal glucose regulation based on evidence from the three vitamin D and type 2 diabetes prevention trials [4–6], and from smaller trials [244–246,248–251] (Table 76.3).

In the D2d study, participants randomized to vitamin D were 31%–45% (depending on the definition of normal glucose regulation used) more likely to have reached normal glucose regulation at their last visit compared with those randomized to placebo. Specifically, when regression to normal glucose regulation was defined as two or three American Diabetes Association glycemic criteria in the normal range (fasting glucose <100 mg/dL, hemoglobin A1c <5.7%, or 2-hour post 75-gram load glucose less than 140 mg/dL) and none in the diabetes range, 12.4% in the vitamin D versus 9.5% in the placebo group had regressed to normal glucose regulation at the last visit over a median follow-up of 2.5 years (rate ratio for vitamin D 1.31; 95% confidence interval 1.02–1.70). When normal glucose regulation was defined as having both fasting glucose and 2-hour post 75-gram load glucose in the normal range (<100 mg/dL and <140 mg/dL, respectively) regardless of hemoglobin A1c, 8.7% of participants in the vitamin D versus 6.0% in the placebo group had regressed to normal glucose regulation at the last visit (rate ratio for vitamin D 1.45; 95% confidence interval 1.05–2.00).

The other two large vitamin D and diabetes prevention trials used slightly different definitions of normal glucose regulation and reported results in the same direction toward benefit; however, results were not statistically significant likely because of smaller sample size compared with the D2d study. In the Tromsø study, normal glucose regulation was defined as having both fasting glucose and 2-hour post 75-gram load glucose in the normal range (<108 mg/dL (6 mmol/L) and <140 mg/dL, respectively) regardless of hemoglobin A1c. By the end of the study, 55 (21.5%) of 256 participants in the vitamin D group and 41 (16.1%) of 255 in the placebo group had achieved normal glucose regulation [5]. In the DPVD study, normal glucose regulation was defined as meeting all three glycemic criteria: hemoglobin A1c <6.5%, fasting glucose concentration <110 mg/dL (6.1 mmol/L), and 2-hour post 75-gram load glucose concentration <140 mg/dL—or both of the following criteria: hemoglobin A1c <5.7% and fasting glucose <100 mg/dL. By the end of the study, 145 (23.0%) of 630 participants in the eldcalcitol

group, and 126 (20.1%) of 626 in the placebo group had achieved normal glucose regulation (hazard ratio 1.15, 95% confidence interval 0.93–1.41) [6].

In addition to the aforementioned three diabetes prevention trials, seven other trials [244–246,248–251] have reported on the effect of vitamin D and regression to normal glucose regulation in people with prediabetes and most have reported favorable effects with vitamin D. In a 1-year trial in the United States among adults with prediabetes and blood 25(OH)D level below 30 ng/mL (mean 22 ng/mL), vitamin D<sub>3</sub> at a mean dose of 88,865 IU weekly in a treat-to-target approach had no effect on rate of regression to normal glucose regulation (38% in vitamin D vs. 41% in placebo) [244]. In an open-label trial in Eastern India among adults with prediabetes and blood 25(OH)D level below 30 ng/mL (mean 17 ng/mL), 60,000 IU weekly of vitamin D<sub>3</sub> for 8 weeks, then monthly for 2 years, improved the likelihood of normoglycemia (42% for vitamin D vs. 20% for placebo) [245]. In a 1-year trial in the United States among Black adults with prediabetes and blood 25(OH)D level below 30 ng/mL (mean 14 ng/mL), vitamin D<sub>2</sub> at 50,000 IU weekly resulted in more participants in the vitamin D returning to normal glucose regulation versus the placebo (32% vs. 8%, respectively) [246]. In a 6-month trial in Iran among adults with prediabetes and blood 25(OH)D levels below 30 ng/mL (mean 13 ng/mL), vitamin D<sub>3</sub> at 50,000 IU weekly resulted in a higher rate of regression to normal glucose regulation (56% for vitamin D vs. 32% for placebo) [248]. In an open-label 78-week trial among overweight/obese Asian Indian women with prediabetes and blood 25(OH)D levels below 20 ng/mL (mean 12 ng/mL), vitamin D<sub>3</sub> at 60,000 IU weekly of vitamin D<sub>3</sub> in a treat-to-target approach improved the likelihood of normoglycemia (59% for vitamin D vs. 29% for placebo based on fasting glucose; 51% for vitamin D vs. 44% for placebo based on 2-hour glucose) [249]. In a 2-year community-based trial in Northern India among rural women with prediabetes and blood 25(OH)D levels below 30 ng/mL (mean 23 ng/mL), vitamin D<sub>3</sub> at 60,000 units per week in a treat-to-target approach increased the number of women with normoglycemia (23 out of 58 for vitamin D vs. 20 out of 58 for placebo) [250]. In a 1-year trial in Greece among people older than 75 years with prediabetes and mean blood 25(OH)D levels of 20 ng/mL, vitamin D<sub>3</sub> at 25,000 units weekly significantly increased the likelihood of participants returning to normoglycemia (17% for vitamin D vs. 0% for placebo) [251].

Overall, the findings from trials show a highly consistent effect of vitamin D in people with prediabetes towards increasing the likelihood of regression to normal glucose regulation.

## 6.5 Vitamin D and regression to normal glucose regulation. Results from metaanalyses

The results from the individual trials on the effect of vitamin D supplementation on regression to normal glucose regulation are consistent with the results from systematic reviews and metaanalyses, which also reported a higher likelihood of reversal to normal glucose regulation with vitamin D. In a metaanalysis using aggregate (study-level) data, Zhang et al. synthesized results from five trials totaling 1080 participants with prediabetes that reported the rate of reversion from prediabetes to normoglycemia [5,244–246,248]. The authors reported a significant benefit of vitamin D for regression to normal glucose regulation by 48% compared with placebo (risk ratio 0.89; 95% confidence interval 0.80–0.99), and results were consistent among different subgroups [257]. The authors concluded that in people with prediabetes, vitamin D increases the reversion rate of prediabetes to normoglycemia.

The metaanalysis by Pittas et al. with individual participant data from the three large randomized, placebo-controlled trials of vitamin D—only among adults with prediabetes [4–6] also assessed whether vitamin D promotes regression to normal glucose regulation. During a median follow-up of 3.0 years, 14.4% of participants in the vitamin D group (cholecalciferol or eldcalcitol combined) compared with 11.1% in the placebo group had regressed to normal glucose regulation at the last study visit (incident rate ratio 1.30; 95% confidence interval 1.16–1.46).

Based on the consistency of results from the metaanalyses using aggregate or individual participant data, when evaluating the overall benefit of vitamin D in prediabetes, the benefit of higher likelihood of reversal to normal glucose regulation should be added to the lower risk of progression to diabetes.

## 7. Optimizing blood 25(OH)D levels and the concept of “treat-to-target”

In observational cohort studies, people with the lowest blood 25(OH)D level have the highest risk of developing type 2 diabetes. However, as noted earlier, such an association may be due to confounding as people with low blood 25(OH)D level have other risk factors for type 2 diabetes, such as poor diet, overweight, and physical inactivity. Therefore, evidence from trials is necessary to test whether people at risk for type 2 diabetes and very low blood 25(OH)D level benefit the most from vitamin D.

In the D2d study, participants with a baseline 25(OH)D level of less than 12 ng/mL, which is the threshold

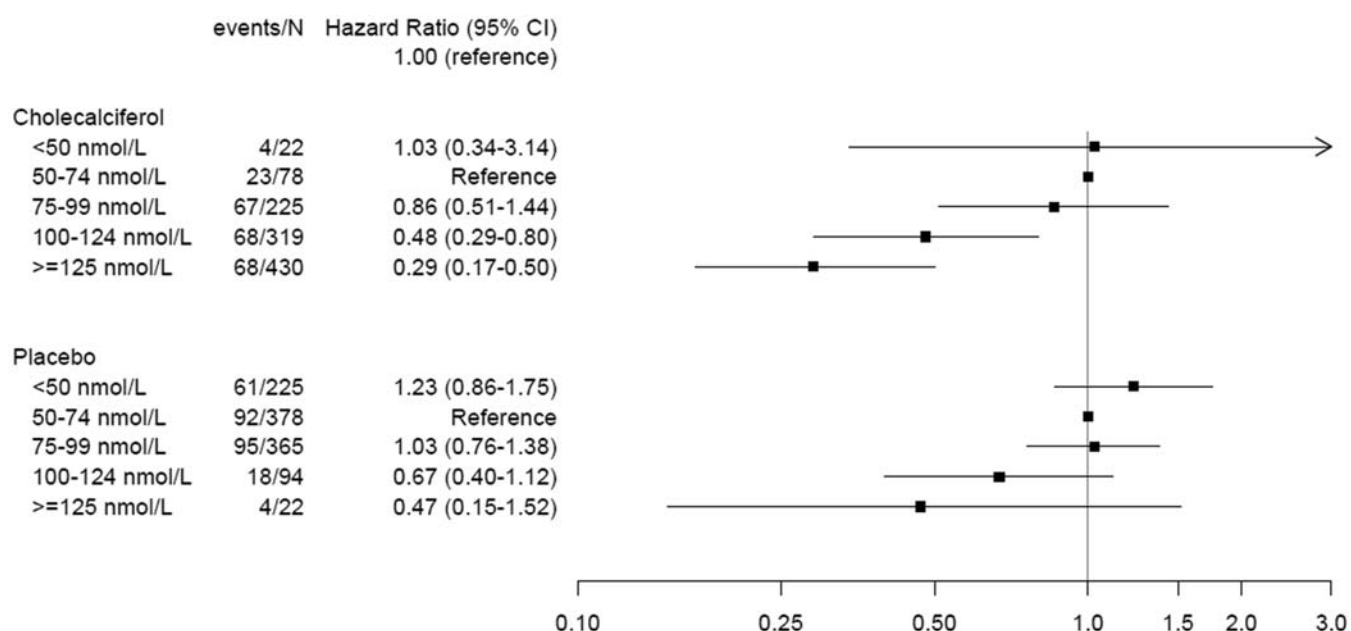
that defines vitamin D deficiency according to the National Academy of Medicine, randomized to vitamin D had a 62% lower risk of developing diabetes compared with placebo (Fig. 76.7) [4]. This result should be interpreted cautiously given the small sample size of the subgroup; however, the effect size was very large, and it is consistent with the dose–response curve expected for vitamins [267] and also consistent with additional evidence from mechanistic studies. In the same subgroup of D2d participants with low baseline 25(OH)D level, vitamin D improved the disposition index [52], indicating a large benefit in beta-cell function among those with very low 25(OH)D levels to begin with, consistent with the clinical result of lower diabetes risk in the same subgroup. Overall, results from observational studies and trials agree that in people with prediabetes, avoiding very low 25(OH)D levels reduces risk of developing type 2 diabetes.

In observational cohort studies, higher blood 25(OH)D levels are associated with lower risk of developing type 2 diabetes, as described earlier. For example, in a nested case–control study design in the NHS cohort of women, those in the top quartile of blood 25(OH)D (mean 33 ng/mL) had a 48% lower risk of developing diabetes compared with those in the bottom quartile (mean 14 ng/mL) [170]. Spline regression models showed a progressive decrease in risk within the higher 25(OH)D range ( $\sim 50$  ng/mL) with no apparent threshold for the relationship between 25(OH)D level and incident diabetes (Fig. 76.3). In a longitudinal observational study in the Diabetes Prevention Program (DPP) among participants with prediabetes, blood 25(OH)D levels were measured annually, and an integrated measure of intrastudy vitamin D status value for each participant was calculated as the predictor variable [193]. Participants in the DPP study who maintained a mean intrastudy blood 25(OH)D level of 30 ng/mL had 28% lower risk of developing diabetes compared with those who maintained intrastudy blood 25(OH)D levels of 13 ng/mL. There was no apparent threshold for the relationship between 25(OH)D level and incident diabetes, and the shape of the curve also suggested a stronger decrease in diabetes risk for 25(OH)D levels above  $>50$  ng/mL although very few participants had that level. Findings in the NHS and DPP cohorts are fully concordant with those from other observational longitudinal studies that have reported on the association between higher blood 25(OH)D and lower risk of developing type 2 diabetes. When combined such data in a metaanalysis, results show that higher 25(OH)D levels ( $>40$  ng/mL) conferred lower risk of developing diabetes, with no apparent plateau for benefit (Fig. 76.4) [189].

The results from the observational studies raise the possibility that in addition to avoiding low blood 25(OH)D levels, achieving and maintaining high blood 25(OH)D levels would optimize diabetes risk reduction. In the D2d study, repeated measures of blood 25(OH)D throughout the trial provided a unique opportunity to test whether achieving and maintaining high intratrial vitamin D levels influenced risk of developing diabetes. For each participant, intratrial vitamin D exposure was calculated as the mean of all annual serum 25(OH)D values prior to the occurrence of the primary endpoint of new-onset diabetes, start of a diabetes or weight-loss medication, or last follow-up visit. Participants who stopped taking their study pills and those who took vitamin D outside of the study were included in this analysis. Thus, this analysis captured incomplete adherence to the assigned treatment and use of off-protocol vitamin D. Categories of participants defined by their achieved intratrial 25(OH)D level were established using National Academy of Medicine cutoffs, and hazard ratios for diabetes among participants in each intratrial 25(OH)D category were estimated compared with the 20–29 ng/mL category (considered “sufficient” for bone health by the National Academy of Medicine) [268]. Results showed a significant interaction of treatment assignment (vitamin D or placebo) with intratrial 25(OH)D level on risk of diabetes [269]. Among participants treated with vitamin D (Fig. 76.9, upper panel), higher achieved intratrial blood 25(OH)D levels favorably influenced the risk of developing diabetes. In fully adjusted models, participants treated with vitamin D who maintained mean intratrial 25(OH)D levels of 40–49 and  $\geq 50$  ng/mL during follow-up were 52% and 71%, respectively, less likely to develop diabetes compared with those who maintained 25(OH)D levels of 20–29 ng/mL. In the placebo group (Fig. 76.9, lower panel), there was also a pattern of declining risk of diabetes at higher intratrial 25(OH)D levels, but the 95% CIs were wide.

Using the same methodology and individual participant data from the two vitamin D and diabetes prevention trials that administered cholecalciferol (D2d and Tromsø), participants treated with vitamin D who maintained intratrial 25(OH)D levels of 40–49 and  $\geq 50$  ng/mL during follow-up were 62% and 76%, respectively, less likely to develop diabetes compared with participants who maintained levels of 20–29 ng/mL [264]. Among participants assigned to placebo, the hazard ratios for diabetes were not significant by 25(OH)D category.

The dose–response findings in the D2d study and in the combined D2d/Tromsø studies that higher achieved 25(OH)D levels result in better treatment-related reductions in the incidence of diabetes increases the credibility



**FIGURE 76.9** Intra-trial cumulative blood 25(OH)D concentration and new-onset diabetes in the vitamin D and type 2 diabetes (D2d) study.

of the intention-to-treat analyses and strengthens the hypothesis that vitamin D plays a significant role in reducing risk of developing diabetes.

Although trials are expected to be free of confounding when they start because of randomization, biases may emerge during follow-up because of nonadherence to the trial intervention, use of rescue medications (e.g., metformin to prevent diabetes), or differential loss to follow-up. These biases, in turn, lead to postrandomization confounding, which may influence the true estimate of the effect of the intervention. Using the intratrial blood 25(OH)D level rather than the randomized assignment to assess the effect of vitamin D exposure on diabetes risk circumvents these sources of postrandomization confounding.

In total, these results indicate that avoiding vitamin D deficiency is desirable, and achieving higher blood 25(OH)D levels is needed to optimally reduce diabetes risk; the blood 25(OH)D threshold appears to be considerably higher than that recommended by the National Academy of Medicine for skeletal health, 20–29 ng/mL [268].

## 8. Effect modification of vitamin D supplementation by BMI

In the metaanalyses by Zhang et al. [257] and Barbarawi et al. [2], subgroup analyses showed that the benefit of vitamin D was primarily seen when combining data from trials with participants that had a mean baseline BMI in the nonobese category (<30 kg/m<sup>2</sup>) (27%

reduction in Zhang et al. and 32% reduction in Barbarawi et al.). Although these subgroup results in aggregate data metaanalyses are problematic, as noted earlier, the same result was reported in the metaanalysis by Pittas et al. using individual participant data [264]. When combining data from the three vitamin D and diabetes prevention trials, vitamin D (cholecalciferol or eldcalcitol) had a prominent effect among those with BMI <30 kg/m<sup>2</sup> (hazard ratio for vitamin D 0.79; 95% CI 0.66–0.95) while the effect was attenuated among those with BMI 30–34.9 kg/m<sup>2</sup> (hazard ratio 0.90; 95% confidence interval 0.72–1.12), and there was no effect among those with BMI ≥ 30 kg/m<sup>2</sup> (hazard ratio 1.07; 95% confidence interval 0.82–1.39).

This finding that the benefit of vitamin D may be more prominent in people with lower BMI while it is attenuated among those with higher BMI has been shown in other trials with vitamin D for nondiabetes outcomes. For example, in the VITAL study, subgroup analyses suggested differential effects of vitamin D on cancer incidence according to BMI, with normal-weight participants (BMI <25 kg/m<sup>2</sup>) who received cholecalciferol having a lower risk of developing cancer than those who received placebo compared with those with obesity (24% lower risk vs. 17% higher risk, respectively) [270]. Similar results were seen in the VITAL ancillary analyses on fractures. Participants with normal-weight (BMI <25 kg/m<sup>2</sup>) who received cholecalciferol had a lower rate of fractures than those who received placebo compared with those with obesity (7% lower risk vs. 17% higher risk, respectively) [271].



There are several possible explanations for the consistent finding that BMI, which reflects adiposity, modifies the vitamin D effect. Vitamin D is fat soluble, and increased sequestration in adipose tissue is thought to lead to decreased bioavailability in people who are obese [165], requiring a higher dose of vitamin D than patients with normal weight to achieve a similar increase in blood 25(OH)D concentration [110,112,272]. Beyond a “sequestration” effect or simply a “volumetric dilutional” effect, there is also evidence that obesity represses vitamin D bioactivation by CYP2R1 [113], leading to reduced production of 25(OH)D, and that weight loss upregulates CYP2R1 expression [114]. This explanation is supported by further results in the meta-analysis by Pittas et al. When individual participant data were combined from the two trials (Tromsø and D2d) that administered cholecalciferol, which requires activation to 25(OH)D in the liver and elsewhere by CYP2R1, there was a significant interaction by baseline BMI, so that supplementation with cholecalciferol reduced risk of diabetes in participants with a baseline BMI below the median of 31.3 kg/m<sup>2</sup> but less in those with BMI equal to or greater than the median (26% vs. 0%, respectively) [264]. In contrast, in the DPVD trial that used eldcalcitol, an active analog of vitamin D that does not require hydroxylation by CYP2R1 or CYP27B1, there was no effect modification by baseline BMI (*p* for interaction = 0.82) [6,264]. These results suggest that the effect of vitamin D on diabetes risk is mediated via its conversion to 25(OH)D by CYP2R1, which is primarily expressed in the liver but also in multiple other tissues including in pancreatic endocrine cells [115], and subsequently to 1.25(OH)<sub>2</sub>D by CYP27B1 in the kidney and pancreatic endocrine cells. This can explain why cholecalciferol appears to work better in leaner people with prediabetes and intact CYP2R1 bioactivity, but less well in those with overweight/obesity who are unable to fully convert vitamin D to 25(OH)D, thereby reducing the exposure of the beta-cell to the beneficial effects of the fully activated vitamin D molecule. Another explanation specific to the pathophysiology of type 2 diabetes is that a low BMI serves as a proxy for the predominance of a pancreatic beta-cell defect (insulin deficiency) in people with prediabetes who benefit the most with vitamin D, since a key proposed mechanism of vitamin D is improvement in insulin secretion. This explanation is supported by results from the DPVD study, which showed that the benefit of eldcalcitol in reducing risk of diabetes was most apparent in participants with insulin secretion at baseline (hazard ratio for vitamin D 0.41; 95% confidence interval 0.23–0.71).

Finally, pancreatic  $\beta$ -cell autoimmunity, which may be present in more than 10% of people diagnosed with type 2 diabetes, leading to pancreatic  $\beta$ -cell destruction is a pathway towards insulin deficiency among non-

obese people, and high-dose vitamin D may attenuate the autoimmune-mediated pathology. Testing the hypothesis that vitamin D helped predominantly participants with prediabetes and evidence of  $\beta$ -cell autoimmunity would be a compelling area for future research to better identify populations at risk for diabetes that are most likely to benefit from vitamin D.

## 9. Safety of vitamin D supplementation for prevention of type 2 diabetes

When examining the benefit of vitamin D for prevention of type 2 diabetes, potential safety concerns need to be addressed. In each of the three vitamin D and type 2 diabetes prevention trials, protocol-specified adverse events of interest (hypercalcemia, hypercalciuria, nephrolithiasis) were rare, and there were no differences between the vitamin D and placebo groups [4–6]. Detailed data from the D2d study support the safety of cholecalciferol 4000 IU per day in overweight/obese participants at high risk for diabetes who were appropriately monitored for safety. Overall, a total of 8304 adverse events occurred during 3 years of follow-up and were less frequent in the vitamin D group compared with placebo (incidence rate ratio 0.94; 95% CI 0.90, 0.98). The overall frequency of protocol-specified adverse events of interest, which included nephrolithiasis, hypercalcemia, hypercalciuria, or low estimated glomerular filtration rate, was low and did not differ by group. There were no significant between-group differences in total serious adverse events. The metaanalysis by Pittas et al. that combined individual participant data from the three large randomized, placebo-controlled trials of vitamin D—only supplementation among adults with prediabetes also assessed safety [264]. During a median follow-up of 3.0 years, the incidence of kidney stones, hypercalcemia, hypercalciuria, and death from any cause did not differ between the groups.

## 10. Conclusions

Results from longitudinal observational studies provide strong support of an association between vitamin D status and development of type 2 diabetes, supported by biological plausibility raising the possibility that optimizing vitamin D status has role when caring for patients with or at risk for type 2 diabetes. The results from mostly underpowered trials and *post hoc* analyses of large trials that used small doses of vitamin D do not support a role of vitamin D for treatment of established type 2 diabetes or prevention of diabetes among those with normal glucose tolerance. In people with prediabetes, vitamin D reduces risk of progression to diabetes and promotes

regression to normal glucose regulation. For optimal risk reduction, blood 25(OH) levels higher than those recommended by the National Academy of Medicine are needed.

## 11. Summary points

- Vitamin D plays a role in pancreatic beta-cell function, insulin action, and systemic inflammation providing mechanistic links between vitamin D and risk of type 2 diabetes.
- Conventional longitudinal observational studies report highly consistent inverse associations between blood 25(OH)D concentration and risk of developing type 2 diabetes. However, confounding is a key limitation.
- Mendelian randomization studies do not support linear associations between genetically determined blood 25(OH)D levels and risk of type 2 diabetes; however, Mendelian randomization studies center upon assumptions that are not relevant to vitamin D physiology.
- In people with normal glucose tolerance or those at average risk for type 2 diabetes, there is no evidence that vitamin D improves glucose tolerance or reduces risk of developing diabetes.
- In people at risk for type 2 diabetes (prediabetes), vitamin D reduces risk of progression from prediabetes to diabetes.
- In people at risk for type 2 diabetes (prediabetes), vitamin D promotes regression to normal glucose regulation.
- For optimal glycemic outcomes in people at risk for type 2 diabetes, blood 25(OH) levels higher than those recommended by the National Academy of Medicine are needed.
- The effect of vitamin D on prevention of type 2 diabetes is attenuated in those with overweight or obesity. Higher doses of cholecalciferol or the use of activated metabolites may be needed for adults with obesity.
- At doses given in the diabetes prevention trials in people with prediabetes, vitamin D is safe.

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# Liver metabolism and disease

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## OBJECTIVES

Vitamin D is well known for its role in calcium and skeletal homeostasis, and it exerts pleiotropic biological functions.  $1\alpha,25$ -Dihydroxyvitamin D<sub>3</sub> ( $1,25(\text{OH})_2\text{D}$ ), the active form of vitamin D derived from  $1\alpha$ -hydroxylation of 25-hydroxyvitamin D ( $25(\text{OH})\text{D}$ ), mediates its function mainly through binding to D receptor (VDR). The  $1,25(\text{OH})_2\text{D}$ –VDR complex regulates its target genes expression by binding to vitamin D response elements (VDREs) in target genes. In the liver, VDR is expressed highly in nonparenchymal cells, including KCs (Kupffer cells), HSCs (hepatic stellate cells), and cholangiocytes, whereas in the hepatocytes, its expression is relatively low.  $1,25(\text{OH})_2\text{D}$ –VDR signaling has been reported to inhibit liver inflammation and fibrosis and antiproliferative effects. Altered  $1,25(\text{OH})_2\text{D}$ –VDR signaling is commonly related with the occurrence, natural course, and outcome of chronic liver diseases. In this chapter, we will mainly discuss the role of  $1,25(\text{OH})_2\text{D}$  and VDR signaling in liver inflammation, fibrosis, and nonalcoholic steatohepatitis (NASH).

## 1. Introduction

The liver is renowned for its essential roles in preserving and regulating the levels of lipid and glucose in the body and energy metabolism. Hepatocytes are responsible for disparate metabolic tasks of energy storage and supply. Hepatic steatosis results from the imbalance between hepatic lipid supply and

utilization [1–3]. Nonalcoholic fatty liver disease (NAFLD) is defined as the accumulation of lipids in the liver in the absence of secondary causes, such as excess alcohol consumption and other chronic liver diseases. NAFLD is a complex liver disease with conditions varying in hepatic lipid deposition, inflammation, hepatocytes injury, and the associated fibrosis. Among these, simple hepatic steatosis (fatty liver) is referred to as NAFL, and nonalcoholic steatohepatitis (NASH) is defined as a more aggressive condition with inflammation and fibrosis. NASH may progress to adverse hepatic outcomes, including cirrhosis, liver failure, and hepatocellular carcinoma (HCC) [4]. NAFLD is strongly associated with obesity and type 2 diabetes mellitus (T2DM) and has become the most common cause of chronic liver disease with a worldwide prevalence of 25%. In the United States, the number of NAFLD cases is projected to expand from 83.1 million in 2015 to 100.9 million in 2030 [5]. The more aggressive NASH will rise from 20% to 27% of adults in these NAFLD cases. As NAFLD/NASH progresses, hepatic inflammation and fibrosis develop, which increases the risk of severe systematic liver diseases, including cirrhosis and HCC [6,7]. This rising liver metabolism-associated disease prevalence will exact a growing economic burden on patients with cirrhosis and end-stage liver disease, including HCC requiring liver transplantation. Over the past decades, great effort has been dedicated to understanding the mechanisms of NAFLD pathogenesis; however, identifying therapeutic targets and advancing drug development are still significant unmet challenges, and no agent is available yet for NAFLD or NASH. Therefore, identifying predictive biomarkers of disease risk, key pathogenic drivers, and novel therapeutic targets are urgently needed.

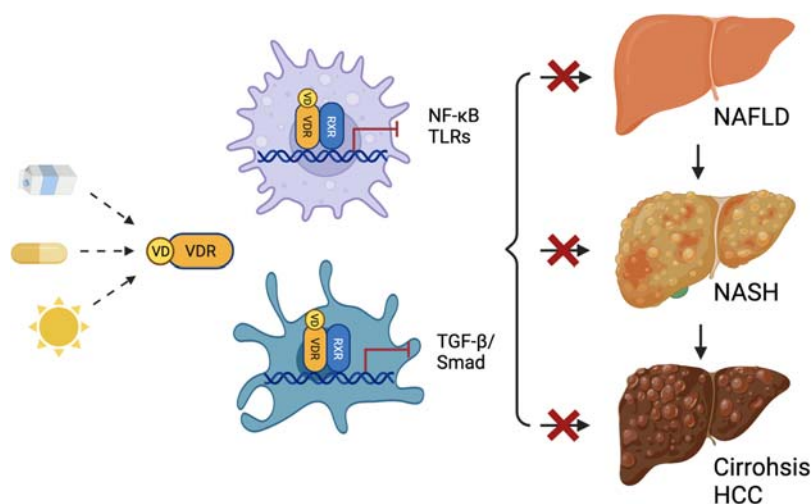
The liver has several routes of lipid acquisition: On one hand, enhanced lipid uptake resulting from white adipose tissue (WAT) insulin resistance induces circulating fatty acid (FA) flux to the liver with subsequent ectopic hepatic lipid deposition. On the other hand, hepatic insulin resistance and excess dietary fructose drive *de novo* lipid synthesis (DNL). It is estimated that dysregulated hepatic lipid uptake accounts for more than half of fatty liver in NAFLD patients [8]. Several free fatty acid transporters, including CD36, fatty acid transport protein 2 (FATP2), and FATP5, have been identified for their function in liver lipid uptake. Hepatocyte-specific ablation of CD36 ameliorates high-fat diet (HFD)–induced NAFLD in mice [9], whereas ablation or knockdown of liver FATP2 or FATP5 alleviates liver steatosis [10,11]. Several nuclear receptors, including peroxisome proliferator–activated receptor- $\gamma$  (PPAR $\gamma$ ), pregnane X receptor (PXR), TAK1/TR4, and aryl hydrocarbon receptor (AHR), directly upregulate CD36 expression and promote liver steatosis [12–15]. PPAR $\gamma$  also promotes hepatic lipid storage by inducing the expression of lipid droplet proteins, whereas hepatocyte-specific deletion of PPAR $\gamma$  decreases HFD-induced hepatic steatosis [16,17]. Hepatic lipid uptake correlates with the release of free fatty acids (FFAs) from white adipose tissue, facilitating lipid trafficking from adipose tissue to the liver during the course of NAFLD pathogenesis [18]. In addition to circulating fatty acids, endoplasmic reticulum (ER) stress–induced hepatic very-low-density lipoprotein receptor (VLDLR) protein expression leads to hepatic steatosis by increasing lipoprotein delivery to the liver [19]. Hepatic *de novo* lipogenesis (DNL) contributes to about 11%, 19%, and 38% of intrahepatic triglyceride (TAG) in the lean, obese, and obese-NAFLD humans, respectively [20]. Dietary sugars, including glucose and fructose, are potent inducers of hepatic lipid synthesis and contribute to NAFLD progression (52, 138). Glucose is metabolized into fatty acids through a chain of reactions mediated by glycolysis, glucose oxidation (TCA cycle), and fatty acid synthesis enzymes. Subsequently, the long-chain fatty acids (LCFAs) are activated to generate LCFA-CoA, providing substrates for triacylglycerol (TAG) synthesis.

The mechanism(s) responsible for increasing hepatic DNL in individuals with NAFLD is not completely understood. Although glucose metabolism is dysregulated with whole-body insulin resistance, hepatic DNL is induced by elevated insulin through activating two master DNL genes, including the sterol regulatory element–binding protein 1c (SREBP-1c) and carbohydrate response element–binding protein (ChREBP), and by the lipogenic transcription factor liver X receptor (LXR) [21–23]. This phenomenon implies differential insulin actions during insulin resistance—suppressing

hepatic glucose production but preserving insulin sensitivity with respect to the SREBP-1c pathway that stimulates fatty acid synthesis. This differential regulation could be at least partially explained by the activation of mTORC1 signaling by excess insulin, which regulates SREBP1c mRNA expression and processing [24,25]. SREBP1c is also regulated independently of insulin, as demonstrated by induction of SREBP1c after feeding in the liver-specific insulin receptor knockout mice [26]. Pharmacological inhibition of DNL, for example, using acetyl-CoA carboxylase inhibitors (MK-4074 and PF-05221304), decreases liver steatosis in patients with NAFLD [27]. Recently, overconsumption of food containing high-sugar/high-fructose corn syrup was linked to NAFLD and NASH development in humans. While the glucose-induced DNL pathway is highly regulated, nearly all fructose is metabolized and committed to DNL by the liver and cleared from portal blood. In the liver, fructose is phosphorylated by ketohexokinase to form fructose-1-phosphate (F1P). F1P then generates substrates for DNL and serves as an allosteric activator of glucokinase, which enhances glycolytic flux in hepatocytes [28]. A large fructose load results in the depletion of liver ATP in humans and animals, which may enhance cellular stress and hepatic damage, leading to NASH development [29].

Several epidemiological studies have reported the correlation of vitamin D deficiency (low serum 25(OH)D) or low 25(OH)D with NAFLD and provided evidence that these conditions increased disease risk. Notably, low serum 25(OH)D levels correlate with the severity of steatosis and hepatic inflammation-related damage. Indeed, vitamin D deficiency is very common in people with NAFLD and NASH [30–32]. For example, a meta-analysis of 29 case–control and cross-sectional studies found that NAFLD patients had significantly lower levels of 25(OH)D and were 1.26 times more likely to be vitamin D deficient. NASH patients also had significantly lower levels of 25(OH)D [30]. A recent Korean cohort study also supported that serum vitamin D level is inversely correlated with NAFLD [33]. Although clinical and metaanalysis studies suggest an inverse association between 25(OH)D and the histologic and pathologic severity of NAFLD and NASH, a clear causal nexus between vitamin D signaling and liver diseases is not determined. Besides, there are also conflicting reports suggesting a lack of association between vitamin D levels and NAFLD severity [34] and no efficacy of vitamin D supplementation on NAFLD treatment [35]. Similar contradictory results also show no association between serum vitamin D levels and vitamin D supplement efficacy in T2DM [36].

The steroid hormone form of vitamin D, 1,25(OH) $_2$ D, is best known for its role in calcium and skeletal homeostasis but is also recognized for its nonskeletal functions



**FIGURE 77.1 Schematic representation of vitamin D/VDR signaling on chronic liver diseases.** Vitamin D is obtained mainly through diet sources (oily fish, meat, milk, and others) and supplements. Sunlight exposure is a natural source of vitamin D synthesis. Vitamin D becomes activated in the liver and kidney and then mediates its function mainly through binding to its receptor VDR. VDR is highly expressed in the nonparenchymal hepatocytes including the hepatic macrophages and stellate cells. Activated VDR heterodimerizes with the cofactor RXR. The VDR/RXR complex translocates to the nucleus, binds to its specific genomic sequences (VDREs) in the promoter region, and regulates the transcription of target genes. In Kupffer cells and hepatic macrophages, VDR signaling interferes with NF-κB signaling and inhibits the expression of TLRs to reduce inflammation. On the other hand, VDR signaling results in reduced HSC proliferation through inhibiting TGFβ/Smad signaling, thus contributing to the alleviation of liver fibrosis. Inflammation and fibrosis are hallmarks of disease progression from NAFLD to NASH and NASH-HCC. Therefore, activation of VDR signaling is a promising intervention for obesity-associated chronic liver diseases.

in immunity, inflammation, cell proliferation, and differentiation [37,38] (see **Volume 2** of this book). The biological functions of  $1,25(\text{OH})_2\text{D}$  are primarily mediated through binding to VDR, a member of the nuclear receptor superfamily (see Chapters 10–13). In the healthy liver, VDR is expressed highly in nonparenchymal cells, including Kupffer cells (KCs), hepatic stellate cells (HSCs), and cholangiocytes. In contrast, the remaining epithelial and resident immune cells express relatively low levels of VDR. Several reports have shown that  $1,25(\text{OH})_2\text{D}$  binding to VDR exerts an inhibitory effect on liver inflammation and fibrosis [39–41]. This chapter will focus mainly on the actions of VDR in hepatic immune cells and stellate cells in NAFLD and NASH (Fig. 101.1).

## 2. Vitamin D/VDR signaling and antiinflammatory responses in the liver

Vitamin D signaling has been well studied for its nonskeletal functions in regulating immunity, inflammation, proliferation, and differentiation [37,38] (see Chapters 94–96). In particular, as part of its antiinflammatory, VDR interacts with IKKβ to interrupt nuclear factor-κB (NF-κB) signaling [42,43]. VDR signaling also reduces Toll-like receptor (TLR)–mediated inflammation in hepatic macrophages and suppresses TLR4-mediated inflammation [44]. At least part of the VDR

antiinflammatory effect is mediated through downregulating miR-155, which derepresses SOCS1, leading to heightened negative feedback regulatory action [45]. As a master regulator of inflammation, NF-κB plays a critical role in the expression of proinflammatory cytokines, such as interleukin (IL)-1β and IL-6. Inactivation of NF-κB signaling protects mice from obesity-induced insulin resistance [46–48]. Thus, VDR activation may improve insulin resistance and T2D through antiinflammatory suppression of NF-κB signaling. Vitamin D–mediated VDR activation promotes macrophage differentiation in vitro and suppresses cytokine secretion in T2D [49]. Deletion of macrophage VDR induces insulin resistance and promotes monocyte cholesterol transport to accelerate atherosclerosis in mice [50]. Suppression of NF-κB activity is the main mechanism for the antiinflammatory function of VDR signaling in hepatic macrophages. Under the NAFLD/NASH condition, liver KCs and the recruited macrophages cross-talk with hepatocytes to regulate inflammation. Hepatic NF-κB activity regulates insulin sensitivity in obesity condition. IKKβ, the key component in the NF-κB cascade, was reported to inhibit insulin sensitivity by inducing IL-1β or IL-6 expression [46,48,51]. We reported that the VDR analog (calcipotriol) suppresses IL-1β, IL-6, and P65 expression in the HFD-induced mouse fatty liver [41]. This supports that VDR activation suppresses liver inflammation to regulate insulin sensitivity and diabetes. Wei et al. reported that VDR regulates β-cell inflammation and sur-



vival through switching the SWI/SNF (BAF) chromatin remodeling complexes [52]. Inhibition of the interaction of VDR-BRD9 (bromodomain-containing proteins) combined with activation of VDR signaling cooperate to dismiss the BAF-BRD9 complex and shift the balance to the activating PBAF-BRD7 complex form to induce a coordinated transcriptional response. Pharmacological activation of VDR signaling by calcipotriol in combination with a BRD9 inhibitor was able to partially restore  $\beta$ -cell function and glucose homeostasis in T2D mouse models. As a consequence, NAFLD condition is also ameliorated [52]. Endoplasmic reticulum (ER) stress occurs when ER homeostasis is disturbed with accumulation of unfolded/misfolded protein or calcium depletion. Under pathological condition, ER stress is associated with liver inflammation, fatty liver development, and fibrosis [53,54]. The antiinflammatory functions of macrophage VDR signaling have also been described in the context of hepatic ER stress resolution in normal chow and HFD-fed mice. Specifically, VDR activation suppresses chemical and diet-induced hepatic ER stress response; VDR knockout mice exhibited persistent unfolded protein response, resulting in hepatic cell death and inflammation and progression of liver disease [55]. In line with this, vitamin D supplementation can improve chronic low-grade inflammation and diabetic condition in T2D patients [56–58]. Therefore, targeting VDR signaling highlights a great potential in ameliorating chronic metabolic diseases. Recently, in a randomized, double-blind, placebo-controlled trial ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT04038853) NCT04038853) found that low-to medium-dose vitamin D supplementation (1000 IU/day) over 12 months reduces the transient elastography indices of liver steatosis and fibrosis in unselected adult patients with NAFLD [59]. In another study, treatment with 2100 IU vitamin D daily over 48 weeks showed a significant improvement of serum ALT levels in patients with hypovitaminosis D and histology-proven NASH. As for NAFLD, primary endpoint showed a trend toward reduction of hepatic steatosis, however, not significant due to a small number of available biopsy specimens [60].

### 3. Vitamin D/VDR signaling in liver fibrosis and NASH

In regard to chronic liver diseases, including liver fibrosis and NASH, HSCs are the major effector cells in the pathogenic wound healing process, leading to liver fibrosis. HSCs provide the predominant source of hepatic extracellular matrix. VDR is highly expressed in HSCs, albeit at lower levels than KCs. VDR activation by 1,25(OH)<sub>2</sub>D in HSCs strongly antagonizes TGF $\beta$  signaling, the most potent profibrogenic pathway in

the liver. This leads to the suppression of a broad range of profibrotic genes, including collagens, integrins, and tissue inhibitors of metalloproteinase [61,62]. Moreover, mouse model studies reveal that spontaneous liver fibrosis occurs when one or both *Vdr* alleles are knocked out, with more severe fibrosis observed in the *Vdr* null mice. More importantly, vitamin D administration resulted in decreased VDR degradation and attenuation of TGF $\beta$ 1-induced fibrosis. Ding et al. demonstrated a genomic circuit between VDR and SMAD to compete TGF $\beta$  signaling. Interestingly, SMAD-mediated TGF $\beta$  signaling enhanced the accessibility of ligand-bound VDR with these genomic loci, which in turn antagonized the recruitment of SMAD3 [62]. Another study addressed the amelioration of hepatic inflammation, fibrosis, and HCC in HSCs through *P62* binding to VDR, which promotes VDR signaling in HSCs and facilitates the stabilization of the VDR/RXR heterodimer [63]. Therefore, it is of note that *P62* levels should be monitored when designing clinical approaches involving targeting VDR in liver fibrosis, NASH, and associated HCC. Given recent data revealing the importance of activated myofibroblasts to NASH and tumor promotion, it is of great interest to investigate detailed mechanisms and the potential of clinical usage of HSC VDR actions during NASH and associated HCC development.

Targeting VDR signaling opens a novel therapeutic window for NAFLD, NASH, and NASH-associated HCC. However, conflicting results from different clinical cohorts puzzled the development of VDR-targeted intervention. One caveat comes from the different forms of vitamin D and its synthetic analogs used in different studies and their various efficacy in activating VDR. The other challenge is the side effect of VDR ligands in elevating serum calcium levels (hypercalcemia). Until now, “noncalcemic” VDR ligands are still in development. Specifically, Dong et al. utilized low-dose calcipotriol administration to achieve therapeutic efficacy in the HFD-induced obesity mouse model without inducing elevated serum calcium level or toxicity [41]. Hepatic macrophages are an attractive target for novel therapies to treat liver steatosis and insulin resistance. KCs and monocytes-derived macrophages have high scavenging capacity, highlighting the potential for precise drug delivery. Evidence from mouse models and human clinical studies supports the notion that the pathogenic macrophage subsets can be successfully targeted under the NASH and liver fibrosis conditions, supporting novel treatment avenues in the future. Clinically relevant carrier materials such as microbubbles, liposomes, and polymers can be detected in the liver, and KCs are the main cellular target for these materials [64]. Dexamethasone coupled with mannosylated albumin can selectively deliver antiinflammatory drugs to the KC,

reducing intrahepatic reactive oxygen species (ROS), inflammation, and fibrosis [65,66]. Besides, emerging fields of medical applications such as nanomedicine may hold exceptional potential for novel therapeutic approaches targeting liver macrophages in liver disease [67,68]. Therefore, the current controversial therapeutic effect of vitamin D in NAFLD might be greatly improved by novel drug carrier materials delivering 1,25(OH)<sub>2</sub>D or its synthetic analogs to the hepatic macrophages to control liver inflammation, NAFLD, and NASH. Targeted delivery of therapeutics to the activated HSCs is critical for successfully treating chronic liver diseases. Many fibrogenic markers, such as type VI collagen receptor, RBP receptor, PDGFR $\beta$ , synaptophysin, IGFIR, LDLR, and CD44, have been identified for activated HSCs [69]. Their ligands have been used to specifically deliver various antifibrotic agents. Despite great efforts in developing targeted delivery systems for liver fibrosis in preclinical studies, precise targeted drug delivery has not been successful in clinical trials. Among clinical trials testing small molecules, proteins, monoclonal antibodies, and nucleic acids in liver fibrosis, one clinical trial (NCT02227459) has used vitamin A as a targeting ligand. Vitamin A-based nanoparticle delivery can successfully deliver antifibrotic siRNAs, miRNAs, or drugs into activated HSCs and reduce the expression of profibrotic genes. Hepatic fibrosis is a consequence of the accumulation of inflammatory cytokines and numerous ECM proteins in the damaged liver. Therefore, targeting one protein expression via gene silencing may not produce efficient results, which is one of the main limitations of siRNA-based therapeutics. However, with combined vitamin D treatment, the activated VDR signaling may provide promising therapeutic effects in the future NAFLD and NASH treatments.

#### 4. Conclusions

Vitamin D is a molecule exerting beneficial effects at several sites of biological homeostasis, besides its central role in bone and mineral homeostasis. Extensive research over the past decades has pointed 1,25(OH)<sub>2</sub>D as a key player in the regulation of liver inflammation and fibrosis. Vitamin D–VDR axis modulates signaling pathways that control antiproliferative, antiinflammatory, and antifibrotic functions in VDR expressing cell types in the liver. In support, the epidemiological evidence of an association between hypovitamin D and the presence of NAFLD and NASH reinforces the rationale of aiming to restore optimal vitamin D levels as a therapeutic intervention for the management of NAFLD and NASH. The measurement of blood 25(OH)D concentration allows personalization

of the supplementation regimen and monitoring of the safety of the treatment throughout the treatment period. Currently, the overall number of randomized clinical trials investigating the effects of vitamin D supplementation in NAFLD and NASH is very limited. Due to high risk of bias and insufficient power of the included trials, further investigations with appropriate study design may be needed to define the benefit of effect of vitamin D supplement on the NAFLD and NASH.

#### 5. Summary points

- Vitamin D by binding to VDR modulates signaling pathways involving antiproliferative, antiinflammatory, and antifibrotic functions in liver nonparenchymal cells.
- Vitamin D deficiency associates with the incidence of NAFLD and NASH in patients.
- Vitamin D/VDR signaling shows beneficial effects in the treatment of NAFLD and NASH in mouse models and some clinical human studies.
- Further clinical investigations with appropriate study design are required to draw the conclusion of whether vitamin D supplementation can serve as an effective intervention in NAFLD and NASH.

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# Vitamin D, hypertension, and cardiovascular disease\*

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## OBJECTIVES

- It has long been known that supraphysiological vitamin D doses cause vascular calcification. More recently, evidence has accumulated that vitamin D may also exert beneficial effects on the cardiovascular system. Therefore, the potential link between vitamin D deficiency and CVD risk has been rigorously studied.
- Since CVD is a leading cause of morbidity and mortality globally, and an inadequate vitamin D status is a worldwide issue as well, the preventive role of vitamin D with regard to the risk of CVD will be the focus of this chapter.
- Several lines of evidence regarding vitamin D and CVD will be summarized. Available data come from experimental animals, patients with rickets or osteomalacia, large prospective cohort studies, genetic association studies, and randomized controlled trials.
- Since harmful vitamin D effects on the cardiovascular system are an issue as well, potential benefits of vitamin D will be weighted against potential harm.

- Finally, conclusions are drawn regarding the practical consequences of the results.

## 1. Introduction

About a hundred years ago, it was discovered that rickets, a disease involving inadequate mineralization of the growing skeleton, and which was endemic in children in North America and Europe at that time, was mainly caused by vitamin D deficiency [1]. The rapid establishment of vitamin D prevention measures could effectively erase this endemic. However, both experimental animal studies and data in children also soon showed that those vitamin D doses, which are now considered supraphysiological, can lead to massive calcification of soft tissues such as vessels and kidneys [2,3]. For this reason, the adverse effects of vitamin D on the cardiovascular system have long been the focus of interest [4–6], and it had been suggested that damage of the cardiovascular system by high doses of vitamin D may predispose for atherosclerosis [4,6]. In line with this assumption, the approach of high-dose intermittent administration of vitamin D for the prevention of rickets

\* **Short note:** Meanwhile, the statistical approach of the two non-linear Mendelian randomization studies mentioned in this chapter [68,77] has been questioned [Smith GD. *Lancet Diabetes Endocrinol.* 2023;11:14]. Indeed, a recalculation of the data by the lead authors of one of the two articles revealed a null effect instead of an increased CVD risk at deficient 25(OH)D levels [Burgess et al. *Lancet Diabetes Endocrinol.* 2023;11:15–6]. Consequently, there is currently no good evidence that even deficient circulating 25(OH)D concentrations increase the risk of CVD.

(six doses of 600,000 IU vitamin D<sub>2</sub> divided over the first 1.5 years of life), which was performed for decades, especially in parts of Germany, led to very high 25-hydroxyvitamin D (25(OH)D) concentrations in the circulation (several hundred and up to more than one thousand nmol/L) and, in one-third of the cases studied, also to hypercalcemia [7]. This strategy of rickets prevention has also been associated with premature death in some children [8].

In the early 1980s, Robert Scragg, taking ecological data as his basis, was one of the first to focus on the association of vitamin D deficiency with cardiovascular disease (CVD). He suggested that vitamin D deficiency may contribute to the risk of CVD [9]. His assumption was based on (1) the circannual rhythm in cardiovascular mortality with a peak in winter and a nadir in summer, (2) the fact that these fluctuations cannot be adequately explained by suggested risk factors such as temperature or respiratory infections, and (3) seasonal variations in solar ultraviolet B radiation, and thus human vitamin D status, parallel the observed CVD findings. The vitamin D deficiency hypothesis is also in agreement with differences in CVD risk by geographic latitude, altitude, and place of residence (rural or urban) [10].

Altogether, the aforementioned data indicate a biphasic vitamin D effect on the cardiovascular system with detrimental consequences from both deficiency and intoxication.

During the past decades, the effects of vitamin D on the cardiovascular system and CVD outcomes have been rigorously studied. CVD includes various illnesses, among them hypertension, heart failure, myocardial infarction, and stroke. Several lines of evidence regarding vitamin D and CVD are now available, such as knockout models in experimental animals, large prospective cohort studies, genetic association studies, and randomized controlled trials (RCTs). Since CVD is a leading cause of morbidity and mortality globally, and an inadequate vitamin D status is a worldwide issue as well, the preventive role of vitamin D with regard to the risk of CVD has been the focus of these recent studies and is also the focus of this article.

## 2. Vitamin D metabolism and regulation with special reference to cardiovascular disease

Circulating 25(OH)D is the generally accepted indicator of human vitamin D status. 25(OH)D is produced in the liver from cutaneously synthesized or orally ingested vitamin D. After hepatic hydroxylation, 25(OH)D is rapidly released into the circulation. According to the Institute of Medicine [11], values < 30 nmol/L are classified as deficient, between 30 and 50 nmol/L as inadequate, between 50 and 125 nmol/L as adequate, and

>125 nmol/L as potentially harmful. For adults, the Institute of Medicine considers daily vitamin D doses of 600–800 as adequate, and the upper tolerable intake level has been set at 4000 IU daily [11]. Nevertheless, there is an ongoing debate about the classification of vitamin D status according to circulating 25(OH)D, and an Endocrine Society recommendation considers a concentration of 75 nmol/L as target value and values up to 250 nmol/L as safe [12]. The corresponding recommended daily intake of the Endocrine Society is 1500–2000 IU, and the safe upper intake level is set at 10,000 IU.

Besides this ongoing discussion, the use of circulating 25(OH)D as an indicator for vitamin D status is also complicated by the fact that 25(OH)D is only the precursor of the active hormone, 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), which is synthesized primarily in the kidney, but also in extrarenal tissues. Renal synthesis of 1,25(OH)<sub>2</sub>D is usually tightly regulated by parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF23) to maintain plasma calcium and phosphate within their relatively narrow physiological ranges (see Chapter 8). FGF23 binds to its cell surface receptor with much higher affinity in the presence of a cofactor, called klotho. FGF23 inhibits 1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase or CYP27B1) and activates 24-hydroxylase (24-OHase or CYP24A1) (see Chapter 19), resulting in decreased 1,25(OH)<sub>2</sub>D and increased 24,25(OH)<sub>2</sub>D levels [13], whereas 1 $\alpha$ -OHase is activated by PTH, as a consequence of low plasma calcium levels [14] (see Chapter 17). At circulating 25(OH)D concentration <30 nmol/L, however, renal 1,25(OH)<sub>2</sub>D synthesis becomes also substrate-dependent. Consequently, deficient vitamin D (serum 25(OH)D) status is often associated with low circulating 1,25(OH)<sub>2</sub>D concentrations and secondary hyperparathyroidism (reference range of PTH: 10–60 pg/mL), while PTH is only slightly elevated and 1,25(OH)<sub>2</sub>D only slightly decreased (but may also be normal or relatively high) at circulating 25(OH)D between 30 and 50 nmol/L [15].

PTH excess is linked to endothelial dysfunction [16] and arterial hypertension [17]. In chronic kidney disease (CKD), secondary hyperparathyroidism and elevated phosphate levels seem to contribute to the high prevalence of vascular calcification and CVD [18] (see Chapter 79). With respect to FGF23, extremely high (several thousand research units (RU)/mL; reference range: <100 RU/mL) concentrations have been described in diseases with low circulating 1,25(OH)<sub>2</sub>D concentrations, such as chronic kidney disease and end-stage heart failure [19–21]. There is evidence that FGF23 activates the renin–angiotensin–aldosterone system (RAAS) and leads to cardiac left ventricular hypertrophy by a calcineurin- and nuclear factor of activated T cells (NFAT)–mediated process [22,23]. Thus, FGF23 is a good predictor of heart failure outcome [24,25]. FGF23 is also a good

predictor of cardiovascular and all-cause mortality [26,27], with a gradual and progressive rise in the risk of all-cause mortality as FGF23 increases [27] (see Chapter 19).

### 3. Vitamin D effects on cardiovascular cells

There is growing evidence that vitamin D signaling has important functions in endothelial cells, vascular smooth muscle cells (VSMC), and cardiomyocytes [28]. The effects of vitamin D in cardiovascular cells are based on its active hormone,  $1,25(\text{OH})_2\text{D}$ , which is either incorporated from the circulation via a membrane-bound vitamin D receptor (VDR) or formed from its precursor  $25(\text{OH})\text{D}$  by an intracellular  $1\alpha\text{-OHase}$  [29]. Thus, it appears likely that  $1,25(\text{OH})_2\text{D}$  can act in an endocrine, autocrine, and paracrine fashion on the cardiovascular system. The effects of  $1,25(\text{OH})_2\text{D}$  are mediated by non-genomic and genomic actions, thereby regulating intracellular calcium metabolism by the rapid release of ionized calcium from intracellular stores or by calcium-binding protein (CaBP) synthesis [28]. Similar to the control of circulating  $1,25(\text{OH})_2\text{D}$ , the synthesis of  $1,25(\text{OH})_2\text{D}$  in the heart and the vasculature seems to be regulated by PTH and FGF23 [30,31].

The effects of  $1,25(\text{OH})_2\text{D}$  on the vascular system appear to be a double-edged sword: several beneficial effects such as a reduction in thrombogenicity, a decrease in vasoconstrictors, an inhibition of oxidative stress and atherogenesis, an improvement in endothelial repair, a reduction in foam cell formation, and vascular relaxation and dilatation have been reported [32,33]. In addition, a lack of  $1,25(\text{OH})_2\text{D}$  promotes transdifferentiation of VSMCs into osteoblast-like cells by suppression of IL-4, IL-10, Matrix-gla protein, osteopontin, osteoprotegerin, and fetuin A and may thus lead to vascular calcification [34].  $1,25(\text{OH})_2\text{D}$  is also considered to be a negative endocrine regulator of the RAAS [35], whose activation plays a pivotal role in hypertension and other CVDs. A lack of vitamin D action by selective deletion of vitamin D receptors (VDR) in endothelial cells increases the sensitivity of the vascular wall to angiotensin II, thereby increasing systemic blood pressure [32]. However, elevated circulating  $1,25(\text{OH})_2\text{D}$  may also contribute to essential hypertension by facilitating calcium transport from the extracellular space into the cell [36], leading to increased smooth muscle cell contractility. In addition, elevated  $1,25(\text{OH})_2\text{D}$  concentrations can promote vascular calcification either by direct hypercalcemic (plasma calcium  $>2.6$  mmol/L) and hyperphosphatemic (plasma phosphate  $>1.61$  mmol/L) effects or by transdifferentiation of VSMCs into osteoblast-like cells [34,37]. In line with this, children on dialysis show a

U-shaped distribution of carotid intima-media thickness and calcification scores across circulating  $1,25(\text{OH})_2\text{D}$  levels: patients with both low and high  $1,25(\text{OH})_2\text{D}$  concentrations in the circulation have significantly greater carotid intima-media thickness and calcification than those with normal levels [38]. The potentially adverse effects of  $1,25(\text{OH})_2\text{D}$  on the cardiovascular system are also underlined by findings that vascular calcification is mediated by an increase in  $1\alpha\text{-hydroxylase}$  expression in VSMCs [39], and loss-of-function mutations of either FGF23 or klotho can induce ectopic calcification [40,41].

### 4. Vitamin D effects in experimental animals

Various rodent models have been used to study the effect of vitamin D on the cardiovascular system in vivo. The models include feeding diets with deficient or excess vitamin D content, generating uremic animals with low circulating  $1,25(\text{OH})_2\text{D}$  concentrations, disrupting vitamin D pathways by deletion of (1) the *Cyp27b1* gene encoding for the  $1\alpha\text{-OHase}$ , (2) the global *Vdr*, or (3) the cardiomyocyte-specific *Vdr*, and generating animals with  $1,25(\text{OH})_2\text{D}$  overexpression.

In normal mice, vitamin D deficiency stimulates renin synthesis, whereas the injection of  $1,25(\text{OH})_2\text{D}$  reduces renin synthesis [35]. Diets with deficient vitamin D content also stimulate osteoblast-like cell formation of VSMCs and aortic calcification in experimental mice [42,43]. In experimental uremia, secondary hyperparathyroidism and hyperphosphatemia are associated with vascular calcification [44]. In  $1\alpha\text{-OHase}$  (*Cyp27b1*) knockout mice, administration of  $1,25(\text{OH})_2\text{D}$  can prevent an upregulation of the RAAS in renal and cardiac tissue and the development of hypertension, cardiac hypertrophy, and impaired cardiac function. These vitamin D effects are independent of plasma calcium and phosphate concentrations [45].

Deletion of the gene for VDR (*Vdr*) in mice has also been reported to elevate production of renin and angiotensin II, leading to hypertension and cardiac hypertrophy [46,47]. Moreover, treatment of VDR knockout mice with the angiotensin-converting enzyme inhibitor captopril reduced cardiac hypertrophy and normalized atrial natriuretic peptide expression [47]. Cardiomyocyte-specific deletion of the VDR resulted in cardiac hypertrophy as well, and treatment of neonatal cardiomyocytes with  $1,25(\text{OH})_2\text{D}$  was partially able to suppress hypertrophy [48]. In addition, deletion of VDR stimulated osteoblast-like cell formation of VSMCs and aortic calcification [42,43]. VDR knockout mice also displayed increased thrombogenicity [49]. It has, however, been criticized that the so-called “rescue diet” enriched with calcium, phosphate, and lactose, usually



administered to normalize mineral homeostasis in VDR knockout mice, is not sufficient to prevent secondary hyperparathyroidism. An excessive dietary calcium concentration was required to reduce serum PTH concentrations in the VDR knockout mice to PTH levels measured in wild-type mice. This diet, however, resulted in higher concentrations of circulating FGF23. Considering that PTH and FGF23 exert numerous VDR independent effects, it has been argued that data obtained from VDR knockout mice cannot be attributed solely to vitamin D [50]. The proposed vitamin D effects on the cardiovascular system have also been challenged by findings that despite increased renin concentrations in mice with VDR deletion, the expression of renin and angiotensin II receptors, blood pressure, heart rate, and heart function remained unaltered [51]. Mice with knockout of the gene for VDR (*Vdr*) are discussed in greater detail in Chapter 30.

Vitamin D intoxication, induced by either excess vitamin D doses or administration of (supraphysiologic) doses of  $1,25(\text{OH})_2\text{D}$  to uremic rats, results in hypercalcemia, hyperphosphatemia, massive increase in aortic calcium and phosphate content, and vascular calcification [18]. Moreover, in mice, genetic inactivation of either FGF23 or *klotho* leads to increased serum concentrations of  $1,25(\text{OH})_2\text{D}$ , calcium, and phosphate, and vascular calcifications. Importantly, vascular calcification can be completely eliminated in either FGF23- or *klotho* knockout mice by additional  $1\alpha\text{-OHase}$  (*Cyp27b1*) deletion. This also results in a change from severe hyperphosphatemia to hypophosphatemia [52]. Vascular calcification is also prevented in *klotho*- and sodium/phosphate cotransporters double knockout mice, in which serum levels of phosphate are normalized, but  $1,25(\text{OH})_2\text{D}$  and calcium levels are still elevated [52]. Data support the assumption that the effect of  $1,25(\text{OH})_2\text{D}$  on serum phosphate is crucial for vascular calcification. Results are also in line with the inhibition of atherosclerotic plaque calcification in VDR deficient mice [53], but challenge the effect of experimental vitamin D deficiency on vascular calcification.

Altogether, available data indicate that deficient vitamin D action results in adverse effects on the cardiac system and may result in vascular calcification by stimulation of osteoblast-like cell formation of VSMCs. Excess vitamin D action also results in vascular calcification, a process that is probably mediated by elevated serum phosphate concentration. Nevertheless, some inconsistencies remain, since the effect of VDR deletion on blood pressure and heart function has been questioned [51]. Thus, caution is necessary in extrapolating results from VDR knockout animals to apparently healthy humans with deficient vitamin D status.

## 5. Rickets, osteomalacia, and cardiovascular disease

A number of case reports have associated nutritional rickets with dilated cardiomyopathy, a specific type of heart failure, but not with other forms of CVD, such as hypertension, stroke, or myocardial infarction. Hypocalcemia (plasma calcium  $<2.2$  mol/L) is considered to be an important cause of heart failure in nutritional rickets, and both the cardiac and skeletal effects can effectively be cured by vitamin D and calcium administration [54,55]. In a cohort of 148 neonates with vitamin D deficiency [56], however, echocardiographic assessment was unable to detect signs of heart failure, indicating that heart failure is a rare complication even in vitamin D-deficient infants, or may only be the result of longer lasting vitamin D deficiency. Heart failure, hypertension, or other forms of CVD have also not been described in genetic forms of rickets based on a lack of the  $1\alpha\text{-OHase}$  enzyme or a deletion of the VDR, although these diseases present with hypocalcemia as well [57,58].

Likewise, CVD is not a leading illness in nutritional osteomalacia, although the disease often results in hypocalcemia in the long run [59]. One exception is the case report of nutritional osteomalacia in a middle-aged woman with hypocalcemia and heart failure, and prompt improvement of the cardiac symptoms on correcting the hypocalcemia by vitamin D and calcium [60]. Anyhow, no cases of CVD have been reported in other forms of osteomalacia, such as tumor-related osteomalacia, either. This rare endocrine disorder occurs predominantly in middle-aged adults and is characterized by hypophosphatemia, phosphaturia, and inappropriately low serum levels of  $1,25(\text{OH})_2\text{D}$ . The biochemical changes are related to elevated FGF23 concentrations [61,62].

Collectively, data in children and middle-aged adults indicate that heart failure is a rare illness in severe vitamin D deficiency. Other forms of CVD such as stroke, hypertension, and myocardial infarction are primarily diseases of the aging population, and children and middle-aged adults are probably not a good model for studying the effect of vitamin D deficiency on these diseases.

## 6. Vitamin D status and cardiovascular outcomes in observational studies

Observational studies provide the opportunity to study the association of vitamin D status with CVD risk in large to very large cohorts. Almost all of these studies use the circulating  $25(\text{OH})\text{D}$  concentration as an indicator of vitamin D status. Several metaanalyses (MAs) of prospectively studied cohorts, case-control,

or cross-sectional studies have analyzed the association of circulating 25(OH)D with the risk of hypertension in adults (Table 78.1). Quantitative results show that the risk of incident hypertension decreases by 7%–12% per 25 nmol/L increment in 25(OH)D levels (64,65,67). Results of the most recent MA (67) also indicate an approximate L-shaped correlation between circulating 25(OH)D levels and hypertension risk in the general population. The data are based on prospective cohort studies and include 43,320 participants with 8397 incident cases of hypertension, mostly from Europe and North America. They also show that hypertension risk below 75 nmol/L increases significantly with decreasing 25(OH)D, especially at levels below 50 nmol/L, and also remains significant over the 75–130 nmol/L range [63]. Overall, the risk of hypertension is about 30% higher at circulating 25(OH)D concentration of 25 nmol/L than at 25(OH)D concentration of 75 nmol/L (67).

Various MAs of cohort studies have also analyzed the association of circulating 25(OH)D with other types of CVD, such as stroke, myocardial infarction, ischemic heart disease, heart failure, and CVD mortality (Table 78.2). The MAs are based on up to 850,000 individuals and report nonlinear associations of circulating 25(OH)D with CVD risk. In detail, compared with a circulating 25(OH)D concentration around 75 nmol/L, CVD risk increases at 25(OH)D concentrations below 50 nmol/L and is highest at concentrations below 25 nmol/L. Notably, several of the results presented in Table 78.2 are based on individual participant data [64,65,67,68], and in 1 MA, results are also based on standardized 25(OH)D concentrations [67]. Moreover, results of the aforementioned MAs are adjusted for several other known risk factors of CVD. According to a recent individual participant data MA with a very large number (>400,000) of individuals without a history of CVD [68], the risk of CVD morbidity and CVD mortality are about 16%–36% and 67%, respectively, higher at circulating 25(OH)D concentration <25 nmol/L than at 25(OH)D concentration ≥75 nmol/L. Thus, the data

indicate that circulating 25(OH)D is an independent predictor of CVD risk. Nevertheless, we should keep in mind that cohort studies are prone to unexplained confounding, which prevents the inference of a causal relationship between vitamin D status and CVD based on this type of study.

## 7. Genetic studies on vitamin D and cardiovascular outcomes

Gene polymorphisms can influence vitamin D metabolism and activity at different stages (Fig. 78.1). The presence of these gene variants can be used to study vitamin D effects on clinical outcomes. Genetic studies have the advantage that they are unaffected by lifestyle factors. Therefore, these studies are less susceptible to confounding and reverse causality bias than observational studies. Genetic studies are located at the interface between traditional observational epidemiology and interventional trials.

### 7.1 Mendelian randomization studies

Mendelian randomization (MR) is an analytical method that uses genetic variants as instrumental variables for modifiable risk factors, such as circulating 25(OH)D that potentially affect CVD risk. About seven to eight genes with single-nucleotide polymorphisms (SNPs) have been identified, which affect circulating 25(OH)D and do not have pleiotropic effects [32]. Genetic studies indicate that gene polymorphisms in 7-dehydrocholesterol reductase, vitamin D-binding protein (DBP), hepatic cytochrome P450 family 2 subfamily R member 1 (CYP2R1, also known as vitamin D-25-hydroxylase), and cytochrome P450 family 24 subfamily A member 1 (CYP24A1, also known as 24-OHase) explain up to 8 nmol/L of the variations in serum 25(OH)D [69,181]. Although this effect is lower than the effect achievable by oral vitamin D

**TABLE 78.1** Meta-analyses of observational studies regarding the risk of hypertension by circulating 25-hydroxyvitamin D concentration.

References	Outcome	Number of individuals	25 (OH)D reference category	25 (OH)D category	Relative risk (95% CI)
Burgaz [172]	Hypertension	70,028	Highest category	Lowest category	1.37 (1.19–1.59)
Kunutsor [173]	Hypertension	4,965	Top third	Bottom third	1.43 (1.16–1.72)
Qi [174]	Hypertension	46,310	>75 nmol/L	<75–50 nmol/L	1.09 (1.05–1.14)
				<50 nmol/L	1.24 (1.08–1.41)
Zhang [63]	Hypertension	43,320	75 nmol/L	50 nmo/L	1.09 (1.04–1.17)
				25 nmol/L	1.30 (1.11–1.52)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval.

**TABLE 78.2** Meta-analyses of observational studies regarding the risk of nonfatal and fatal cardiovascular events by circulating 25-hydroxyvitamin D concentration.

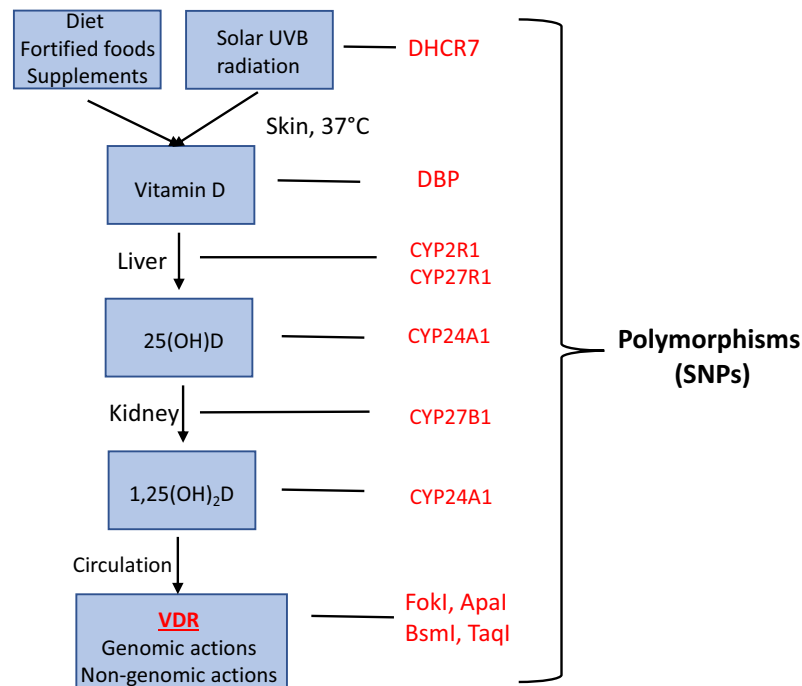
References	Outcome	Number of individuals	25 (OH)D reference category	25(OH)D category	Relative risk (95% CI)
Wang [64]	Cardiovascular disease	65,994	80 nmol/L	50 nmol/L	1.21 (1.00–1.42)
				25 nmol/L	1.94 (1.58–2.32)
Brodum-Jacobsen [175]	Ischemic stroke	58,384	≥75 nmol/L	<25 nmol/L	1.36 (1.09–1.70)
Schöttker [65]	CVD mortality without CVD history	23,081	Top quintile	Bottom quintile	1.41 (1.18–1.68)
	CVD mortality with CVD history	2706	Top quintile	Bottom quintile	1.65 (1.22–2.22)
Chowdhury [176]	CVD mortality	849,412	Top third	Bottom third	1.14 (1.01–1.29)
Fan [177]	CVD mortality	20,937	Highest category	Lowest category	1.57 (1.24–2.00)
Zhang [66]	CVD events	180,667	75 nmol/L	50 nmol/L	1.00 (0.95–1.05)
				25 nmol/L	1.08 (1.04–1.15)
	CVD mortality	85,669	75 nmol/L	50 nmol/L	1.04 (0.95–1.61)
				25 nmol/L	1.15 (1.05–1.22)
Gaksch [67]	CVD mortality	26,916	75–99.99 nmol/L	40–49.99 nmol/L	1.65 (1.39–1.97)
				<30 nmol/L	2.21 (1.50–3.26)
Zhang [178]	CVD mortality	7551	—	Per 25 nmol/L decrease	1.41 (1.27–1.59)
Yang [179]	CVD mortality	21,079	>75 nmol/L	25–50 nmol/L	1.16 (1.04–1.27)
				<25 nmol/L	1.47 (1.15–1.81)
Gholami [180]	CVD mortality		Highest category	Lowest category	1.54 (1.29–1.84)
Sofianopoulou [68]	Stroke	427,698	≥75 nmol/L	25–49 nmol/L	1.10 (1.01–1.19)
				<25 nmol/L	1.36 (1.24–1.49)
	Coronary heart disease	417,937	≥75 nmol/L	25–49 nmol/L	1.00 (0.94–1.06)
				<25 nmol/L	1.16 (1.04–1.29)
	CVD mortality	431,489	≥75 nmol/L	25–49 nmol/L	1.22 (1.19–1.26)
				<25 nmol/L	1.67 (1.48–1.88)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CI, confidence interval.

supplements or skin synthesis of vitamin D, genetic studies have the important advantage over other study types that they reflect lifelong differences in circulating 25(OH)D.

Some publications using the MR approach have investigated the association of genetically determined 25(OH)D with CVD outcomes [68–76]. They used a focused choice of genetic variants (that is, variants in gene regions related to vitamin D synthesis and metabolism) and used one [74] to four [68,75,76] SNPs to perform the analyses. Another MR [77] used a polygenic choice of genetic variants (that is, all genome-wide significant predictors of 25(OH)D).

An MR analysis showed that 25(OH)D levels were not causally associated with lipid parameters in Chinese or European adults [69]. With respect to blood pressure, neither was strong evidence found for a causal effect of vitamin D status on gestational hypertension or pre-eclampsia [75]. Large MR analyses in male and nonpregnant female adults from cohorts of European ancestry from Europe and North America demonstrated, however, that genetically determined differences in 25(OH)D concentration result in small ( $\leq 0.3$  mmHg), but significant, effects on systolic and/or diastolic blood pressure reduction and an 8% reduction in the risk of hypertension [72,73]. In a large nonlinear MR in individuals of



**FIGURE 78.1** Metabolic pathway of vitamin D with selected enzymes and encoding genes. Several genes encode for enzymes involved in different steps of vitamin D synthesis and metabolism such as 7-dehydrocholesterol synthesis, vitamin D transport in the circulation, 25-hydroxylation, and degradation of 1,25(OH)<sub>2</sub>D. Polymorphisms in these genes may result in reduced or enhanced concentrations of circulating 25(OH)D and 1,25(OH)<sub>2</sub>D, whereas vitamin D receptor polymorphisms potentially influence vitamin D action in target tissues.

European genetic ancestry, curved associations were observed for circulating 25(OH)D with systolic and diastolic blood pressure, with individuals with 25 nmol/L estimated to have 0.70 and 0.25 mmHg higher blood pressure compared with 50 nmol/L [77]. Thus, data indicate small, but significantly beneficial, effects on blood pressure in adults in the long run.

Several MR analyses did not demonstrate causal associations of genetically determined 25(OH)D levels with vascular diseases such as myocardial infarction, stroke, ischemic heart disease, ischemic stroke, subarachnoid hemorrhage, intracerebral hemorrhage, or cardiovascular mortality in Chinese, North American, and European people [69–71,74,76]. However, because the participants of these investigations were adults of different ages, results of many individuals might reflect the association between 25(OH)D and disease progression, rather than disease occurrence. Moreover, the aforementioned MR analyses did not account for baseline vitamin D status. Therefore, data of a very large MR analysis by Sofianopoulou et al. [68] in individuals of European ancestries (from the UK Biobank dataset as well as three smaller datasets) with no known history of coronary heart disease or stroke at baseline are of utmost importance. This investigation reported no associations of genetically predicted 25(OH)D with coronary heart disease, stroke, or cardiovascular mortality in population-wide genetic analyses. Most importantly, however, among

the strata of vitamin D–deficient participants (25(OH)D concentration below 25 nmol/L), genetic analyses provided strong evidence for an inverse association with cardiovascular mortality, and suggestive evidence of inverse associations for stroke and coronary heart disease. In detail, per 10 nmol/L higher genetically predicted 25(OH)D concentration, the chance of CVD mortality, stroke, and CHD was in the vitamin D deficiency strata on average reduced by 31%, 15%, and 11%, respectively. In another nonlinear MR by Zhou et al. [77] that used broadly the same data as the nonlinear MR by Sofianopoulou et al. [68], individuals with circulating 25(OH)D at 25 nmol/L had significantly (11%) higher odds of CVD (composite of coronary artery disease, stroke, and peripheral vascular disease) compared with those with 50 nmol/L. There appeared to be a very slight further lowering in the odds of CVD with higher concentrations, and for example, participants with 75 nmol/L had 2% lower odds compared with 50 nmol/L, indicating beneficial vitamin D effects up to circulating 25(OH)D of 75 nmol/L. Correction of circulating 25(OH)D level below 50 nmol/L was predicted to result in a 4.4% reduction in CVD incidence and further increased to 5.7% at 75 nmol/L [77]. However, there are concerns that the effects on CVD risk may be overestimated [78], since Zhou et al. [77] used a polygenic choice of genetic variants (35 genome-wide significant predictors of 25(OH)D), whereas Sofianopoulou



et al. [68] used a focused choice of genetic variants (four gene regions related to vitamin D synthesis and metabolism). Therefore, it has been argued that results in the study by Zhou et al. may be partly attributable to pleiotropic associations of the genetic variants in the analysis, especially in the strata of circulating 25(OH)D above 25 nmol/L [78]. The use of MR in analyzing the potential impact of vitamin D status on human health is discussed in greater detail in [Chapter 61](#).

## 7.2 Polymorphisms in the VDR gene and cardiovascular outcomes

Another group of genetic studies have analyzed the association of variants in genes encoding proteins, which may influence vitamin D metabolism or vitamin D signaling pathways, with CVD risk ([Fig. 78.1](#)). The gene encoding the VDR protein is located on chromosome 12. Several functional VDR SNPs are known: BsmI and ApaI in intron 8, TaqI in exon 9, and FokI in exon 2. In these mutations, the base cytosine is replaced by thymine (FokI), guanine by adenine (BsmI), thymine by guanine (ApaI), and thymine by cytosine (TaqI). A number of metaanalyses reported significant associations of Apa 1, Fok 1, Taq 1, and Bsm 1 polymorphisms of the VDR gene with parameters of CVD risk, such as hypertension, vascular and microvascular complications, or coronary artery disease [79–83]. One MA reported no associations of these gene variants with coronary artery disease [84]. However, none of the aforementioned MAs reported *P*-values for the described associations of  $<10^{-7}$ , which is considered to be the threshold for reliable causal associations of a gene polymorphism with a disease outcome. The numbers of included cases and controls ranged from 2306 to 8011, and from 1635 to 4860, respectively. For genetic studies, these numbers were relatively small. Therefore, it cannot be ruled out that the MAs were statistically underpowered to demonstrate strong associations between VDR polymorphisms and CVD risk.

## 7.3 Polymorphisms in the CYP24A1 gene and cardiovascular outcomes

The CYP24A1 gene is located on chromosome 20. This gene, encoding for the 24-OHase, is responsible for the catabolism of 25(OH)D and 1,25(OH)2D and thus for the regulation of the concentration of these two vitamin D metabolites in the circulation. CYP24A1 is induced by 25(OH)D, 1,25(OH)2D, and FGF23 and is one of the most highly inducible genes in humans, capable of increasing its transcription 20,000-fold [85]. Several genetic variations of the

CYP24A1 gene are known. The association of polymorphisms in the CYP24A1 gene was analyzed in a metaanalysis in three independent populations, including 3657 individuals [86]. In the MA, the C allele (minor allele frequency ranging from 0.25 to 0.31 across the three populations) was associated with lower coronary artery calcification, yielding a *P*-value close to the aforementioned threshold of  $10^{-7}$  ( $2.9 \times 10^{-6}$ ). Moreover, in Chinese people, genetic variations of the CYP24A1 gene were significantly associated with susceptibility to hypertension [87]. However, further functional studies are needed to provide more evidence on how genetic variants of the CYP24A1 gene affect hypertension susceptibility.

## 8. Vitamin D supplementation studies

### 8.1 Vitamin D effects on biochemical risk markers

RCTs provide the highest level of scientific evidence. Studies on biochemical markers can provide insights into short-term and mid-term vitamin D effects regarding CVD risk. These markers include traditional risk markers such as lipid parameters and nontraditional risk markers such as PTH and FGF23 (see earlier sections), inflammation markers, and parameters of the RAAS.

#### 8.1.1 Lipid parameters

Dyslipoproteinemia is a well-known risk factor for CVD. There is a link between cholesterol and vitamin D since the precursor of vitamin D, 7-dehydrocholesterol, can not only be converted by solar UVB radiation to previtamin D<sub>3</sub>, but alternatively also by an enzymatic reaction, mediated by the enzyme 7-dehydrocholesterol-reductase, to cholesterol. A large MA has summarized data from RCTs regarding the effect of vitamin D supplementation on lipid parameters in adults [88]. The average vitamin D dose was 3000 IU/day; two-thirds of the studies had mean baseline 25(OH)D levels  $<50$  nmol/L, and the increase in circulating 25(OH)D was  $48 \pm 23$  nmol/L. Data indicate small, but significant, reductions in total cholesterol, LDL cholesterol, and triglycerides of  $-0.15$  mmol/L,  $-0.10$  mmol/L, and  $-0.12$  mmol/L, respectively, and an increase in HDL cholesterol of  $0.09$  mmol/L by vitamin D supplementation. However, there was no significant vitamin D effect according to baseline 25(OH)D concentration or daily vitamin D dose on lipid parameters. Similar effects of vitamin D supplementation were reported in another MA [89] for total cholesterol, LDL cholesterol, and triglycerides. In contrast to the

aforementioned metaanalysis, a nonsignificant decrease of  $-0.10$  mmol/L was reported for HDL cholesterol. In that MA, the improvements in total cholesterol and triglycerides were more pronounced in participants with baseline 25(OH)D below 50 nmol/L than in individuals above this level [89]. In children and adolescents, vitamin D supplementation did not affect lipid parameters, with the exception of a reduction in triglycerides by 25 mg/dL in the subgroup of individuals with a total vitamin D supplementation  $\geq 200,000$  IU [90]. However, all included four RCTs were very small trials, so the results need to be confirmed by larger studies.

### 8.1.2 PTH and FGF23

With respect to PTH, it is well known that the effect of vitamin D on this hormone is influenced by various factors such as initial 25(OH)D and initial PTH concentrations, vitamin D dose, frequency of vitamin D administration, concomitant calcium intake, and body weight and composition [91]. An MA of RCTs has demonstrated that in apparently healthy individuals with initial 25(OH)D  $< 50$  nmol/L and  $\geq 50$  nmol/L, vitamin D supplementation suppresses serum PTH on average by 17 pg/mL and 2 pg/mL, respectively, indicating a threshold of PTH concentrations around 50 nmol/L [92]. In patients with heart failure and CKD, the suppression of serum PTH by vitamin D supplementation was on average 13 pg/mL and 32 pg/mL, respectively [93,94], whereas vitamin D supplementation did not significantly influence serum PTH, but resulted in a significant increase in phosphorus levels in patients on dialysis [95]. In another MA in patients with CKD, the effect on the suppression of serum PTH was more pronounced by administration of active vitamin D metabolites or analogs than by native vitamin D supplementation [91]. FGF23 remains unaffected by vitamin D supplementation up to doses of 3000 IU daily, whereas higher daily dose equivalents or activated vitamin D significantly increase FGF23 concentrations [96,97]. The effect of vitamin D administration on FGF23 increment is higher in patients with end-stage kidney/heart failure than in other individuals [96]. Altogether, vitamin D supplementation suppresses PTH levels, but dose-dependently also increases FGF23 concentrations.

### 8.1.3 Renin–angiotensin–aldosterone system

Activation of the RAAS plays a pivotal role in the etiology of hypertension and other CVDs such as heart failure. Due to the high prevalence of these diseases, various drugs targeting the RAAS have been developed, such as renin inhibitors, angiotensin-converting enzyme inhibitors, angiotensin 1-receptor antagonists, diuretics, and mineralocorticoid receptor antagonists (Fig. 78.2). The frequent use of these drugs in the adult population

complicates isolated investigations on the effect of vitamin D on the human RAAS system. Nevertheless, the effect of vitamin D supplementation on parameters of the RAAS (renin, angiotensin II, aldosterone) has been investigated in different patient groups, including patients with insufficient vitamin D status [98–100], non-insulin-dependent diabetes mellitus [101–103], heart failure [104–107], and CKD [108,109]. The vast majority of studies reported no significant effect on parameters of the RAAS. In detail, vitamin D was ineffective in studies investigating patients with insufficient/deficient vitamin D status [98–100] or CKD [108,109]. With respect to heart failure, two studies reported no significant effects [104,105], and one study each reported a significant decrease in plasma renin [106] or aldosterone [107]. In diabetic patients, no effect of vitamin D on RAAS parameters was reported in two studies [101,103] and a decrease in plasma renin in one study [102].

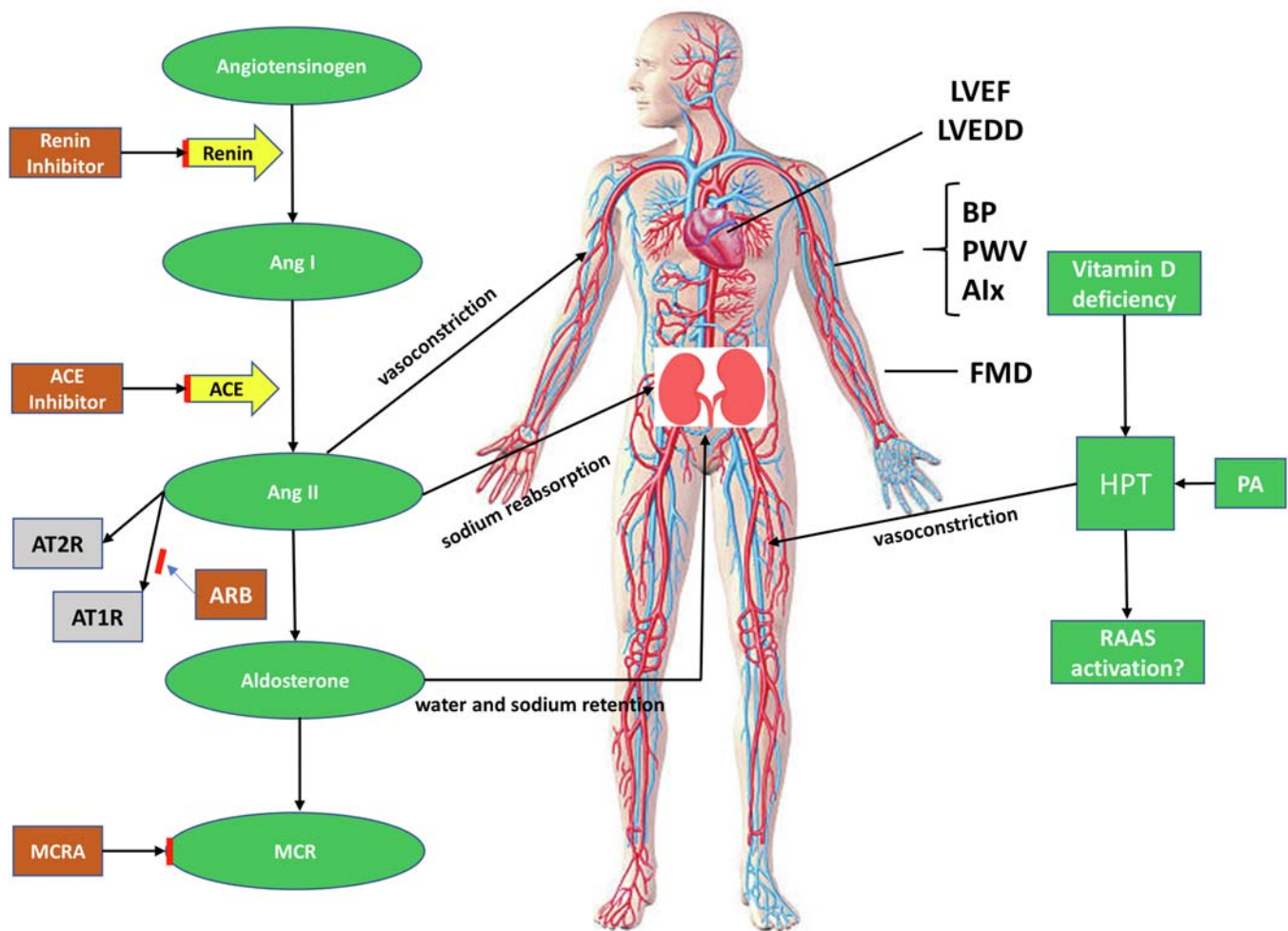
### 8.1.4 Inflammation parameters

With respect to C-reactive protein (CRP), an MA reported a small suppression of  $-0.20$  mg/L by vitamin D supplementation [88]. This effect on CRP seems to be dose-dependent since daily vitamin D doses  $< 1000$  IU had no beneficial effect, whereas doses  $\geq 1000$  IU/d had a favorable effect. Moreover, metaregression indicated that older age predicted a significant decrease in CRP and IL-6 [110]. In patients with HF, a disease resulting in high concentrations of inflammation markers, the effect of vitamin D on inflammation is inconsistent since no significant effect has been reported [111], as well as a suppression in CRP [112]. Moreover, a significant suppression of the proinflammatory cytokine tumor necrosis factor- $\alpha$  was reported, whereas concentrations of interleukin-6 remained unaffected. It was concluded that vitamin D supplementation may have specific, but modest effects on inflammatory markers in this group of patients [111]. Irrespective of some inconsistencies and open uncertainties such as the effect of baseline and achieved circulating 25(OH)D, vitamin D dose, age effects, and disease-specific effects, it is noteworthy that accumulating evidence does not support a causal effect of inflammation on CVD risk [113,114], thereby questioning the use of inflammatory parameters as CVD risk markers.

## 8.2 Vitamin D effects on parameters of cardiovascular function

### 8.2.1 Vascular and cardiac function

Functional parameters represent the interface between biochemical risk markers and clinical events. Vascular function is influenced by the interactions



**FIGURE 78.2** Interrelationships between the renin–angiotensin–aldosterone system (RAAS), parathyroid hormone, and the cardiovascular system. Renin synthesizes angiotensin I (ang I) from angiotensinogen. Ang I is further cleaved to angiotensin II (ang II) by angiotensin-converting enzyme (ACE). Ang II acts on angiotensin 1 and 2 receptors (AT1R, AT2R), resulting in vasoconstriction and renal tubular reabsorption of sodium. Ang II also activates aldosterone secretion. Aldosterone acts on mineralocorticoid receptor (MCR) by retaining water and promoting sodium retention. The RAAS can be blocked at different stages by renin inhibitors, ACE inhibitors, angiotensin receptor blockers (ARBs), and mineralocorticoid antagonists (MCRAs). Primary aldosteronism (PA) results in hyperparathyroidism (HPT). Vitamin D deficiency also results in HPT. HPT promotes vasoconstriction and probably also activates the RAAS by stimulation of renin, ang II, and aldosterone activity. Vascular function can be assessed by the measurement of blood pressure (BP), pulse wave velocity (PWV), augmentation index (AIx), and dilation flow-mediated dilation (FMD). Cardiac function can be assessed by measurement of left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVEDD).

among endothelial cells, VSMCs, adventitial tissues, and inflammatory cells. Vascular function can be assessed by measuring aortic pulse wave velocity (PWV, the velocity at which the blood pressure pulse propagates through the circulatory system) and augmentation index (AIx, the ratio of late systolic pressure to early systolic pressure). Both parameters are accepted measures of arterial stiffness. High PWV and AI values are considered unfavorable. Ultrasonographic measurement of flow-mediated dilation (FMD, dilation of an artery when blood flow increases in that artery following a transient period of forearm ischemia) can be used to assess endothelial function. Low values are considered unfavorable.

Echocardiographic measurements of left ventricular ejection fraction (LVEF) and left ventricular end-diastolic diameter (LVEDD) are frequently used tools to assess cardiac function and the risk of heart failure. LVEF values below 50% indicate a reduced stroke volume of the heart. The reference range for LVEDD is 33–56 mm, whereas CVDs, such as heart failure, are associated with elevated values.

The effect of vitamin D supplementation on vascular and cardiac function has recently been summarized [28]. Trial-level as well as individual-patient-level data showed no consistent effect of vitamin D supplementation on PWV, AI, or FMD, but a beneficial effect of a daily



vitamin D dose  $\geq 2000$  IU on PWV cannot be excluded. Results are based on trials with a duration of 4–57 weeks and daily doses of 600–7100 IU. With respect to cardiac function in patients with heart failure, a dose-dependent beneficial effect was considered on LVEF (+4.2%) and LVEDD (–2.3 mm) in trials with doses of 1000–7100 IU vitamin D daily [28]. A more recent MA supported the beneficial effect of vitamin D supplementation on LVEF in patients with heart failure (+3.3%). Although substantial heterogeneity among trials was reported in that MA, which so far included the largest number of trials and study participants, a dose-dependent effect could not be confirmed in this analysis [115]. It is also noteworthy that heart failure with preserved LVEF accounts for about 40%–50% of incident heart failure cases overall [116]. In this group of patients, the effect of vitamin D on LVEF may be absent or of limited clinical relevance, as well as in individuals without heart failure.

### 8.2.2 Hypertension

Besides the aforementioned vascular and cardiac parameters, blood pressure is another major parameter influencing clinical CVD events [117]. Hypertension (systolic blood pressure  $\geq 140$  mmHg; diastolic blood pressure  $\geq 90$  mmHg) is a risk factor for coronary artery disease, myocardial infarction, heart failure, and cerebrovascular stroke. The aforementioned MA by Mirhosseini et al. [88] included 39 RCTs in their analysis on blood pressure and reported a small, but significant reduction in systolic blood pressure of –0.10 mmHg and of –0.07 mmHg in diastolic blood pressure by vitamin D supplementation. The effects were more pronounced if in-study 25(OH)D  $\geq 86$  nmol/L were achieved, the daily vitamin D dose was  $\geq 4000$  IU, and the duration of intervention was  $\geq 6$  months. Results cover the 95% CI of an MA incorporating individual patient data from 27 RCTs, concluding that vitamin D supplementation is ineffective as an agent for lowering blood pressure [118]. In the latter analysis, trial-level metaregression found no significant relationship between systolic or diastolic blood pressure and daily vitamin D<sub>3</sub> dose equivalent, study duration, baseline PTH concentration, and baseline 25(OH)D. In line with these data, two more recent MAs reported that vitamin D does not influence blood pressure in children and adolescents [90] or the general adult population [63]. However, there is some evidence that in elderly hypertensive patients, vitamin D supplementation reduces systolic and diastolic blood pressure on average by –4.0 mmHg and –2.2 mmHg, respectively [119]. Likewise, there is evidence that vitamin D supplementation may reduce the risk of incident preeclampsia in pregnant women [120], a disease that manifests as hypertension, edema, and proteinuria.

## 9. Cardiovascular events in large vitamin D supplementation trials in general populations

The Holy Grail of evidence-based medicine is an adequately powered RCT with a treatment that can impact a clinically relevant primary endpoint. Four large randomized controlled vitamin D supplementation trials [121–124], focusing on CVD outcomes, such as hypertension or CVD events, have been performed in the general elderly (>50 years, >55 years, >60 or 65 years, and >70 years, respectively) population during recent years. These trials were performed in New Zealand (Vitamin D Assessment trial, ViDA), the United States (Vitamin D And Omega-3 Trial, VITAL), and Europe (Vitamin D3–Omega-3–Home Exercise–HeALTHy Ageing and Longevity Trial, DO-Health; Finnish vitamin D trial, FIND). ViDA, VITAL, DO-Health, and FIND included 5110, 25,871, 2157, and 2495 males and females, respectively, with a median study duration of 3.3 years in ViDA, 5.3 years in VITAL, 5.0 years in FIND, and a treatment duration of 3 years in Do-Health. In the treatment arms, study participants received a monthly bolus dose of 100,000 IU vitamin D<sub>3</sub> (ViDA), 2000 IU vitamin D<sub>3</sub> daily (VITAL, DO-Health), and 1600 or 3200 IU daily (FIND). In all four trials, the control group received a placebo. In the four trials, mean baseline circulating 25(OH)D concentrations were above 50 nmol/L, and mean in-study 25(OH)D concentrations increased to at least 94 nmol/L. Primary endpoints were blood pressure (DO-Health) or a composite of fatal and nonfatal CVD events (ViDA, VITAL, and FIND).

In DO-Health [123], vitamin D supplementation did not significantly influence systolic or diastolic blood pressure values. In ViDA [121], vitamin D supplementation neither significantly influenced the primary endpoint nor secondary endpoints, such as myocardial infarction, angina pectoris, heart failure, hypertension, arrhythmias, arterio sclerosis, stroke, and venous thrombosis. Similarly, in VITAL [122] and FIND [124], vitamin D supplementation neither significantly affected a composite of nonfatal and fatal CVD events nor an expanded composite of major cardiovascular events plus coronary revascularization. In none of the RCTs did results change substantially when analyses were restricted to study participants with baseline 25(OH)D concentrations <50 nmol/L. Unfortunately, however, none of the trials reported outcome data in the subgroup of study participants with baseline 25(OH)D concentrations <25–30 nmol/L, although it is also noteworthy that the prevalence of individuals with such low concentrations was apparently very low in all four trials. The D-Health trial is an ongoing Australian study with 21,315 recruited elderly participants receiving a monthly bolus dose of 60,000 IU vitamin D or placebo over 5 years



for prevention of mortality. Neither all-cause mortality nor CVD mortality was significantly influenced by vitamin D supplementation [125]. This study will also capture nonfatal cardiovascular events through self-reports in annual surveys [126]. However, since baseline 25(OH)D concentrations were on average 77 nmol/L [125], a beneficial vitamin D effect on nonfatal CVD events seems to be unlikely as well.

Independent of frequency and dosing of vitamin D, all aforementioned large trials documented null effects with respect to CVD-related outcome parameters. Results are in general agreement with metaanalyses of RCTs [118,127–130] and an umbrella review of RCTs [131], which included trials where CVD outcomes were only secondary endpoints. However, none of these studies focused on individuals with vitamin D deficiency, i.e., circulating 25(OH)D < 25–30 nmol/L.

## 10. Vitamin D in end-stage organ failure

In CKD and end-stage heart failure, secondary hyperparathyroidism, low 25(OH)D and 1,25(OH)<sub>2</sub>D concentrations, and elevated phosphate concentrations are prevalent [132–135] (see [Chapter 79](#)). In CKD, CVD is a leading cause of overall mortality and a predictor of CVD mortality [135]. Patients with CKD or chronic heart failure enter a vicious circle, named cardiorenal or renocardial syndrome, where functional impairment of one organ affects the function of the other. At present, it remains unclear whether in these diseases the aforementioned changes in the PTH–vitamin D axis are causally related to CVD events.

An MR analysis suggested even harmful actions of vitamin D on kidney function, since it indicated a negative causal effect of log transformed 25(OH)D on log-transformed eGFR. Results showed that a 10% increase in serum 25(OH)D levels causes a 0.3% decrease in eGFR. A significant harmful association was also reported for 1,25(OH)<sub>2</sub>D on eGFR [136]. Although significant, the effect of vitamin D on eGFR was, however, small, and an ancillary study to the VITAL trial in 1312 participants with diabetes mellitus at baseline showed no difference in eGFR changes or albuminuria over 5 years of treatment with vitamin D compared with placebo [137]. These data support the assumption that long-term supplementation of 2000 IU is safe with respect to kidney function. A reanalysis of the extended-release calcifediol trials in CKD patients that used doses of 25(OH)D resulting in very high circulating 25(OH)D concentrations (study duration 26 weeks) found no significant differences in biochemical safety parameters, such as plasma calcium, phosphate, FGF-23, and eGFR, even up to mean circulating

25(OH)D concentrations of 235 nmol/L [138]. With respect to clinical endpoints, observational studies (21 studies, 221,610 patients) actually indicate a risk reduction in CVD mortality of 45% by bolus administration of high vitamin D doses or the administration of activated vitamin D [139]. However, an MA of RCTs (17 studies, 1819 patients) reported only a nonsignificant reduction in CVD mortality of 7% by administration of vitamin D or activated vitamin D [139]. Again, results indicate caution regarding overinterpretation of observational data. In line with this assumption, a relatively large trial [140] in patients with secondary hyperparathyroidism undergoing maintenance hemodialysis (976 patients) reported that the risk of a composite measure of fatal and nonfatal cardiovascular events even tended to be 36% higher in patients receiving activated vitamin D versus usual care, when the per protocol set was analyzed.

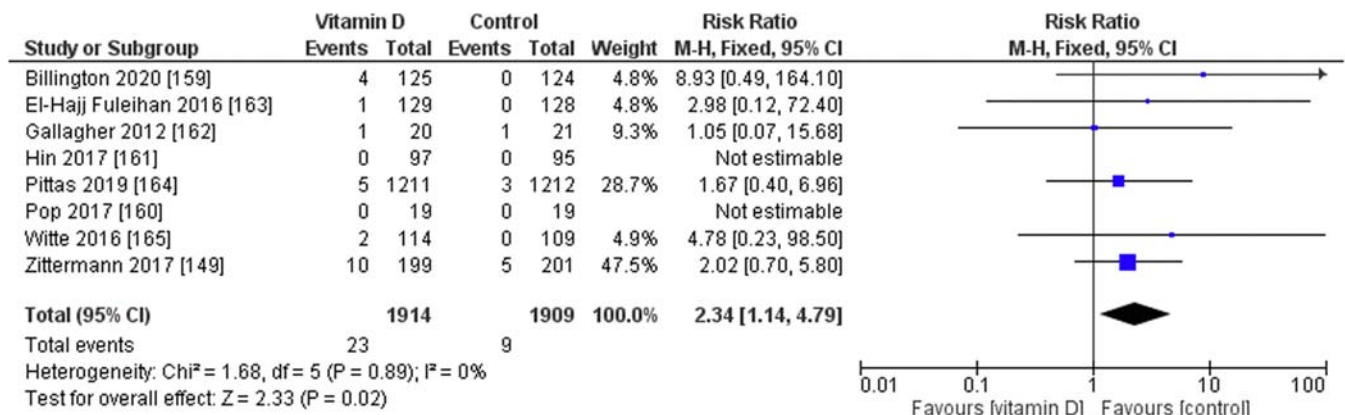
In end-stage heart failure, data regarding the effect of vitamin D supplementation on clinical events are scarce. In patients awaiting heart transplantation, 3 years of daily vitamin D supplementation with 4000 IU did not significantly influence the primary endpoint (overall mortality), but increased the need for mechanical circulatory support implantation [141]. Since the higher risk of mechanical circulatory support implant disappeared in the vitamin D group during a 3-year postintervention follow-up, data support the assumption of adverse vitamin D effects on the cardiovascular system at doses of 4000 IU daily in patients with advanced heart failure [142]. Moreover, vitamin D supplementation of 4000 IU daily did not significantly influence the prevalence of secondary hyperparathyroidism and the concentration of FGF23 [104,143]. Thus, a suggested beneficial effect of vitamin D in advanced heart failure is challenged by these data. Since PTH not only has harmful effects on the cardiovascular system but is also considered to increase myocardial blood flow and cardiac output, it has been suggested that in some patients, high PTH concentrations may, at least in part, be the result of an adaptation process to the severity of the disease, rather than the result of low vitamin D metabolite concentrations [134]. Surprisingly, in end-stage heart failure, low and not high PTH concentrations were predictive of poor clinical outcome [144]. Probably, low PTH concentrations together with low 1,25(OH)<sub>2</sub>D and high FGF23 concentrations prevent patients with failing hearts from more severe derangements in mineral metabolism, such as acute calcium and phosphate intoxication [14]. In addition, immobilization may result in calcium release from bone, leading to a suppression in circulating 1,25(OH)<sub>2</sub>D and PTH concentrations. Overall, it seems that in patients with kidney or heart failure, no simple association exists between vitamin D status, PTH, FGF23, and CVD risk.

## 11. Adverse vitamin D effects on the cardiovascular system

For the general population, the Institute of Medicine has set the upper threshold of adequate circulating 25(OH)D concentrations at 125 nmol/L and the upper tolerable intake level for vitamin D at 4000 IU [11]. In line with the circulating 25(OH)D threshold, individual participant data MA in European individuals, mostly from the general population, reported no significantly increased CVD morbidity or mortality at 25(OH)D concentrations between 100 and 140 nmol/L [67,68]. Likewise, another MA did not provide evidence for an increased risk of CVD events at 25(OH)D levels between 100 and 137 nmol/L in the general population [66]. Similar results were also reported in a rural Chinese population [145]. In contrast, several observational studies in the clinical setting reported a U-shaped or inverse J-shaped association of circulating 25(OH)D with CVD risk [146–149]. Data indicate that the risk of CVD events may already be increased at 25(OH)D levels above 100 nmol/L. The cause of this association remains largely unknown. Potential explanations include the possibility that high 25(OH)D levels (1) reflect low availability of the active vitamin D hormone 1,25(OH)<sub>2</sub>D and may thus be indicative of deficient rather than excess vitamin D action, (2) are related to individuals with the *APOE ε 4* gene, which is associated with an increased CVD risk, and (3) are associated with excess intestinal calcium absorption resulting in hypercalcemia [147].

With regard to the risk of hypercalcemia, a generally accepted indicator of vitamin D intoxication, data are available from some long-term (>1 year) randomized controlled trials on supplementation with 4000 IU of vitamin D daily [141,150–156], a dose considered to be the upper tolerable intake level [11,157]. In these studies,

the weighted mean baseline 25OHD concentration was 62 nmol/L, and the weighted mean increase in the vitamin D versus control group was 61 nmol/L, resulting in mean circulating 25(OH)D concentrations of about 123 nmol/L. The absolute incidence of hypercalcemia (>2.6 mmol/L up to > 2.75 mmol/L, depending on definition) in the vitamin D supplemented and control groups was 1.20% and 0.52%, respectively (Fig. 78.3). Likewise, in another MA of long-term (≥24 weeks) vitamin D supplementation, which included all eligible RCTs, irrespective of the vitamin D dose administered, the absolute incidence of hypercalcemia was in the vitamin D-supplemented groups significantly higher than in the control groups (1.03% vs. 0.54%) [158]. A vitamin D-mediated cause of hypercalcemia are biallelic and, in some instances, monoallelic loss-of-function mutations in the *CYP24A1* gene. The diseases are associated with elevated serum 1,25(OH)<sub>2</sub>D concentrations, suppressed PTH concentrations, hypercalciuria, nephrocalcinosis, and hypertension [159,160]. The prevalence of hypercalcemia-related *CYP24A1* mutations in the general population is unknown [161], but may be about 1:33,000 births in Europe [159]. Overall, even when the upper tolerable intake is not exceeded, vitamin D supplementation appears to increase the risk of hypercalcemia in a small proportion of individuals. The clinical importance of small elevations in serum calcium is highlighted by findings that (1) heart failure incidence increases progressively from serum calcium of 2.25 mmol/L up to 2.75 mmol/L [162] and (2) genetically predicted lifelong higher concentrations of serum calcium may shorten life expectancy and increase CVD risk [163]. Even a low incidence of adverse vitamin D effects may have substantial consequences at a population level. It cannot be ruled out that the effect on prandial or postprandial serum calcium is even more pronounced than on the frequently collected



**FIGURE 78.3** Effect of vitamin D supplementation of 4000 IU daily on the risk of hypercalcemia. Vertical marks represent hypercalcemia risk, and horizontal bars represent 95% CIs; a statistically significant result was assumed when the 95% CI did not include 1 (vertical line). Note that two studies reported no case of hypercalcemia.

fasting blood samples, since a daily vitamin D dose of 4000 IU increases intestinal calcium absorption rate significantly (by about 6%–7%) [164].

Whereas large vitamin D supplementation trials in the general older population with average in-study circulating 25(OH)D concentrations of 104 and 132 nmol/L did not provide evidence for adverse vitamin D effects on the cardiovascular system [120,121], in critically ill heart failure patients, 3-year vitamin D supplementation increased in-study 25(OH)D concentrations to 100–110 nmol/L on average, and also increased the risk of poor clinical outcome significantly [140,165]. Moreover, impaired kidney function is considered to contribute to the loss of homeostatic control of serum calcium concentration and may thus influence the cutoff point defining vitamin D toxicity [11]. Therefore, there continues to be large uncertainty about the progressive health effects of regular ingestion of moderately high amounts of vitamin D in the long term. Altogether, especially in the clinical setting, long-term vitamin D supplementation with moderately high doses should be performed with caution and only based on clear medical indications. Upper tolerable intake levels of vitamin D and adequate circulating 25(OH)D concentrations generated for the apparently healthy general population should not a priori be adopted in the clinical setting.

## 12. Conclusion

Data regarding vitamin D and CVD are puzzling: experimental data demonstrate various beneficial, but also harmful vitamin D effects on the cardiovascular system. Some of these effects may be mediated not only by vitamin D itself, but also by hormones regulating vitamin D metabolism such as PTH and FG23. Large observational studies support an inverse association of vitamin D deficiency with CVD risk. Despite this accumulating evidence regarding detrimental effects of vitamin D deficiency on the cardiovascular system, CVD is, however, rarely seen in patients with nutritional rickets or genetic causes of rickets and osteomalacia, probably because CVD is primarily a disease of the aging population. In addition, the available studies may have been too small to capture the potentially small vitamin D effects on CVD risk. Moreover, there is no strong evidence for beneficial effects of vitamin D supplementation on biochemical risk markers, functional parameters of the cardiovascular system, or CVD events. Nevertheless, in the vast majority of trials, study participation was not restricted to vitamin D deficient adult individuals, i.e., people with baseline initial 25(OH)D concentrations <25–30 nmol/L. Therefore, two large MR investigations [68,77], a study type that

is able to assess lifelong differences in vitamin D status, are of utmost importance, because they indicate that deficient 25(OH)D concentrations increase the risk of CVD morbidity and mortality. Moreover, there is some evidence that gene variants influencing cellular vitamin D action or vitamin D catabolism may influence CVD risk [77–81,83]. However, further studies have to show whether enhanced and/or prolonged cellular vitamin D actions reduce or increase CVD risk. Nevertheless, some data indicate that in future a more personalized vitamin D supplementation advice may become possible [166]. Meanwhile, the entire group of individuals with circulating 25(OH)D concentrations <25–30 nmol/L should be the target population with respect to the prevention of any vitamin D deficiency–related CVD risk. In addition, circulating 25(OH)D concentration above 100–125 nmol/L should be avoided, especially in the clinical setting, to prevent harmful vitamin D effects on the cardiovascular system.

Inadequate vitamin D status is a worldwide issue. The prevalence of vitamin D deficiency (<25–30 nmol/L) in the entire adult population in the United States and Europe is 8% and 13%, respectively, but is considerably higher in Afro-Americans (32%) and non-European immigrants (up to 50% and more) [167,168]. In several low- to middle-income countries, the prevalence of deficient circulating 25(OH)D concentrations is between 40% and 90% in some groups of the general population, such as children, women, and older adults [169]. Even if sun exposure and thus skin synthesis of vitamin D are limited or absent, the officially recommended daily vitamin D doses for adults of 600–800 IU daily should be effective in preventing circulating 25(OH)D concentrations <25–30 nmol/L in the vast majority of individuals [170]. This dose would also be far removed from harmful effects of vitamin D excess on the cardiovascular system. Since results of the aforementioned large MRs [68,77] are based on individuals with no known history of coronary heart disease or stroke at baseline, prevention of vitamin D deficiency should start as early as possible. To erase vitamin D deficiency globally, (mandatory) vitamin D food fortification rather than the (individual) use of vitamin D supplements would be most promising [171].

## 13. Summary points

- Several lines of evidence such as ecological data, experimental animal studies, observational studies, and Mendelian randomization studies indicate a detrimental effect of vitamin D deficiency on the cardiovascular system and CVD outcomes.



- Although studies in patients with rickets or osteomalacia as well as vitamin D supplementation trials were largely unable to support beneficial vitamin D effects on CVD risk, several study limitations may have been responsible for these null effects.
- Individuals with circulating 25(OH)D concentrations <25–30 nmol/L should be the target population for the prevention of any adverse effect of vitamin D deficiency on the cardiovascular system.
- The officially recommended oral vitamin D doses of 600–800 IU should be able to prevent deficient circulating 25(OH)D concentrations in the vast majority of adult individuals.
- Since there is evidence for a U-shaped or inverse J-shaped association of circulating 25(OH)D with CVD risk, especially in the clinical setting, long-term vitamin D supplementation with moderately high doses should be performed with caution and only based on clear medical indications.

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# Vitamin D and renal disease

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## OBJECTIVES

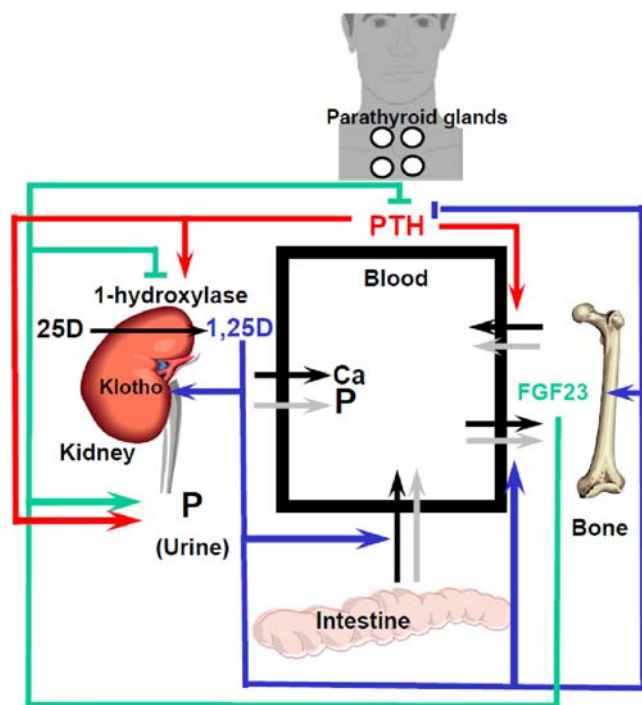
- Present the basic pathophysiological mechanisms underlying CKD-induced vitamin D deficiency and disruption of renal and extra-renal vitamin D bio-activation and catabolism.
- Detail the pro-survival actions of the 1,25(OH)<sub>2</sub>D–VDR complex, which oppose the accelerated aging of the skeletal, immune, renal, and cardiovascular systems in CKD.
- Highlight the role of vitamin D maintenance of the FGF23/klotho axis in avoiding the renal and cardiovascular damage associated with hyperphosphatemia, low serum klotho, high FGF23, and excess active vitamin D in CKD.
- Emphasize the importance of correcting vitamin D deficiency prior to initiation of 1,25(OH)<sub>2</sub>D (or analog) in the clinical setting.
- Discuss current recommendations regarding supplementation strategies to correct vitamin D deficiency with CKD progression.

## 1. Introduction

The vitamin D endocrine system is essential for human health, and a normal kidney is critical to maintain the functional integrity of the vitamin D endocrine system. Large epidemiological studies in normal individuals have demonstrated that vitamin D deficiency is

associated with an increased relative risk for cardiovascular and all-cause mortality [1,2]. In chronic kidney disease (CKD), a disorder affecting more than 10%–12% of the world population, the progressive loss of the capacity of the injured kidney to maintain the functional integrity of the vitamin D endocrine system contributes to accelerated skeletal, immune, renal, and cardiovascular aging, resulting in a 10–20-fold increase in morbidity and mortality compared with gender and age-matched individuals with normal renal function [3,4].

Strong molecular basis for the adverse impact of abnormal kidney function on the vitamin D endocrine system emerged in 1971 with the discovery that the kidney is the main site for the synthesis of 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D or 1,25(OH)<sub>2</sub>D), the hormonal form of vitamin D [5] and the most potent endogenous metabolite to activate the vitamin D receptor (VDR) [6]. In 1984, 1,25(OH)<sub>2</sub>D replacement therapy in advanced CKD patients was utilized to counteract the impaired intestinal calcium absorption and increased synthesis and secretion of parathyroid hormone (PTH). The latter resulted in defective bone mineralization, increased fracture risk, and soft tissue and vascular calcification [7] (see the interactions of the calcium/1,25(OH)<sub>2</sub>D/PTH axis summarized in Fig. 79.1). Over the past 4 decades, studies aimed at overcoming the challenges of effectively controlling secondary hyperparathyroidism (SHPT) with 1,25(OH)<sub>2</sub>D replacement therapy, while avoiding skeletal and cardiovascular risks, have greatly expanded our understanding of the pathogenesis of disrupted vitamin D metabolism and its protective actions in the course of CKD progression. These studies have led to three critical paradigm shifts in our understanding of vitamin D metabolism and



**FIGURE 79.1** Central role of renal calcitriol production in mineral and skeletal homeostasis. Renal 1,25(OH)<sub>2</sub>D (1,25D) production tightly controls the complex hormonal feedback loops between PTH and FGF23/klotho, as well as Ca (black arrows) and P (gray arrows) fluxes between the intestine, bone, and the kidney that ensure normal mineral homeostasis and skeletal integrity, while preventing the excess of both ions predisposing to ectopic calcifications and the proinflammatory, proaging actions of hyperphosphatemia. Kidney disease impairs the renal uptake of 25(OH)D, calcitriol synthesis, and the maintenance of renal klotho causing severe abnormalities in the calcitriol/FGF23-klotho/PTH loops (see text for details) accelerating the development of hyperphosphatemia, bone loss, propensity to fractures, soft-tissue calcifications, renal and cardiovascular damage, and high mortality rates. PTH, parathyroid hormone; FGF23, fibroblast growth factor-23.

actions that continue to shape the clinical application of vitamin D interventions in CKD: (1) The development of parathyroid cell resistance to 1,25(OH)<sub>2</sub>D-mediated suppression of SHPT has required the generation of synthetic 1,25(OH)<sub>2</sub>D analogs with an efficacy similar to that of 1,25(OH)<sub>2</sub>D to suppress PTH but with less calcemic and phosphatemic activity. Importantly, analog treatment conferred hemodialysis patients with a survival advantage over 1,25(OH)<sub>2</sub>D treatment through renal and cardiovascular protective actions not fully accounted for by their efficacy in reducing circulating PTH [8]. (2) Vitamin D deficiency is highly prevalent in CKD patients [9] and associated with a mortality risk higher than that of 1,25(OH)<sub>2</sub>D deficiency [10,11], highlighting an unprecedented role for the kidney in maintaining circulating levels of the 1,25(OH)<sub>2</sub>D precursor 25-hydroxyvitamin D (25(OH)D) and facilitating continued extra-renal 1,25(OH)<sub>2</sub>D synthesis [1]. These findings led to an update in the 2017 KDIGO guidelines to normalize serum 25(OH)D in CKD patients prior to

the initiation of 1,25(OH)<sub>2</sub>D (analog) therapy for SHPT. (3) 1,25(OH)<sub>2</sub>D induces expression of the phosphaturic hormone FGF23 [12] and of the longevity gene  $\alpha$ -klotho [13] to maintain the functional integrity of the FGF23-klotho/1,25(OH)<sub>2</sub>D/phosphate axis, which suppresses PTH and protects against the renal and cardiovascular pro-aging effects of hyper-phosphatemia and hypervitaminosis D and maintains the anti-aging, pro-survival benefits of renal and circulating klotho. However, in CKD, 1,25(OH)<sub>2</sub>D (analog) therapy for secondary hyperparathyroidism (SHPT) may further exacerbate hyperphosphatemia and the increases in serum FGF23, which together with the progressive reductions in renal klotho from early CKD2 generate resistance to FGF23 phosphaturic and PTH suppressive actions. Significantly, high FGF23 exerts a klotho-independent induction of left ventricular hypertrophy (LVH) worsening the risk of cardiovascular mortality [14]. Thus, therapeutic strategies with calcimimetic drugs, which control secondary hyperparathyroidism with attenuated increases in FGF23, have challenged the efficacy of 1,25(OH)<sub>2</sub>D suppression of PTH in reducing cardiovascular risk. However, the recent discoveries of CKD-induced increases in FGF23 unrelated to 1,25(OH)<sub>2</sub>D therapy (reviewed in Refs. [15,16]) as well as of 1,25(OH)<sub>2</sub>D induction of FGF23/soluble klotho/FGFR1 ternary complexes to antagonize FGF23 pathological actions [17] demand a re-examination of current recommendations for vitamin D interventions in CKD. The link between vitamin D and FGF23 is discussed further in Chapter 19.

This chapter updates the progress in our understanding of the molecular pathophysiology underlying CKD-induced disturbances in systemic and tissue-specific vitamin D metabolism and actions that accelerate the onset and progression of SHPT and of skeletal, immune, renal, and cardiovascular aging and compromise survival. This knowledge should help identify tissue-specific targets for 1,25(OH)<sub>2</sub>D/analog pro-survival actions to facilitate the development of vitamin D interventions that safely prevent the onset of CKD or attenuate its progression to end-stage renal disease. Related chapters in this book include Chapter 26 (vol. 1), Chapter 47 (vol. 1) and Chapter 80 (vol. 2).

## 2. Renal regulation of the vitamin D endocrine system

The integrity of the vitamin D endocrine system plays a central role in the tight control of complex calcium and phosphate fluxes, as well as the secretory control and hormonal interactions among the kidney, the parathyroid glands, bone, and intestine [18]. Normal renal synthesis and secretion of the potent calcitropic hormone 1,25(OH)<sub>2</sub>D [19] are critical to integrate the calcium/PTH axis as well as the phosphorus/FGF23-klotho loop

that ensures normal calcium and phosphorus homeostasis and skeletal integrity [20]. These loops are essential in preventing an excess of calcium and phosphate ions that could lead to over-mineralization of bone, ectopic calcification, and also renal and cardiovascular aging driven by hyperphosphatemia, high FGF23, and renal klotho reductions (reviewed in Ref. [21]) and depicted in Fig. 79.1.

Briefly, in normal individuals, a decrease in serum calcium is sensed by the parathyroid gland, which rapidly enhances the secretion and/or synthesis of PTH to restore calcium balance. Elevations in serum PTH induce calcium resorption from bone and stimulate the expression and activity of renal CYP27B1, also called 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase), the enzyme responsible for the conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D. The increases in serum 1,25(OH)<sub>2</sub>D induce intestinal calcium absorption, renal reabsorption, and bone resorption if dietary calcium is insufficient. Upon normalization of calcium levels, both calcium and 1,25(OH)<sub>2</sub>D close the loop by suppressing PTH synthesis and, in the case of 1,25(OH)<sub>2</sub>D, also through a tight regulation of its own circulating levels by suppressing renal 1 $\alpha$ -hydroxylase expression and activity and by inducing its own catabolism by renal CYP24A1 (24-hydroxylase) to avoid the toxicity associated to hypervitaminosis D [19].

1,25(OH)<sub>2</sub>D also elevates serum phosphorus levels by promoting intestinal absorption, renal reabsorption, and bone resorption, increases that promote vascular calcification in a PTH-independent manner [22]. Both high phosphorus and 1,25(OH)<sub>2</sub>D, the latter only in the presence of hyperphosphatemia and a certain calcium threshold [23], induce FGF23 stabilization [24] and synthesis in bone, respectively [12,15]. In the normal kidney, a highly coordinated regulation of renal CYP27B1 and CYP24A1 expression and activity by serum 1,25(OH)<sub>2</sub>D, PTH, FGF23, calcium, and phosphate ensures the functional integrity of skeletal and mineral homeostasis [25].

1,25(OH)<sub>2</sub>D also induces the renal expression of the longevity/antiaging klotho molecule, whose transmembrane and soluble forms are critical for the proper functioning of the phosphorus/1,25(OH)<sub>2</sub>D/FGF23–klotho loop required for the prevention of hyperphosphatemia and hyper-vitaminosis D-induced skeletal, renal, and cardiovascular aging [26]. Specifically, the FGF23/klotho loop exerts a direct inhibition of 1 $\alpha$ -hydroxylase expression, induces CYP24A1, the enzyme responsible for 1,25(OH)<sub>2</sub>D degradation, and also lowers serum phosphorus, through the inhibition of both renal phosphate reabsorption [27,28] and PTH secretion, thereby attenuating bone resorption and the risk of vascular calcification. Circulating klotho not only acts as a co-receptor for FGF23 signaling but also exerts skeletal, renal, and cardiovascular protection in an FGF23-independent manner [29].

In CKD, a defective maintenance of circulating 25(OH)D by the injured kidney aggravates the

progressive loss of the renal capacity to synthesize 1,25(OH)<sub>2</sub>D. The combined vitamin D and 1,25(OH)<sub>2</sub>D deficiency exacerbates the early reductions in circulating klotho, severely compromising the functional integrity of the PTH/1,25(OH)<sub>2</sub>D/calcium and FGF23-klotho/1,25(OH)<sub>2</sub>D/phosphate axes. Appropriate regulation of these axes is essential to control SHPT, to counteract the skeletal, renal, and cardiovascular aging driven by hyperphosphatemia and to protect from excessive FGF23-driven LVH.

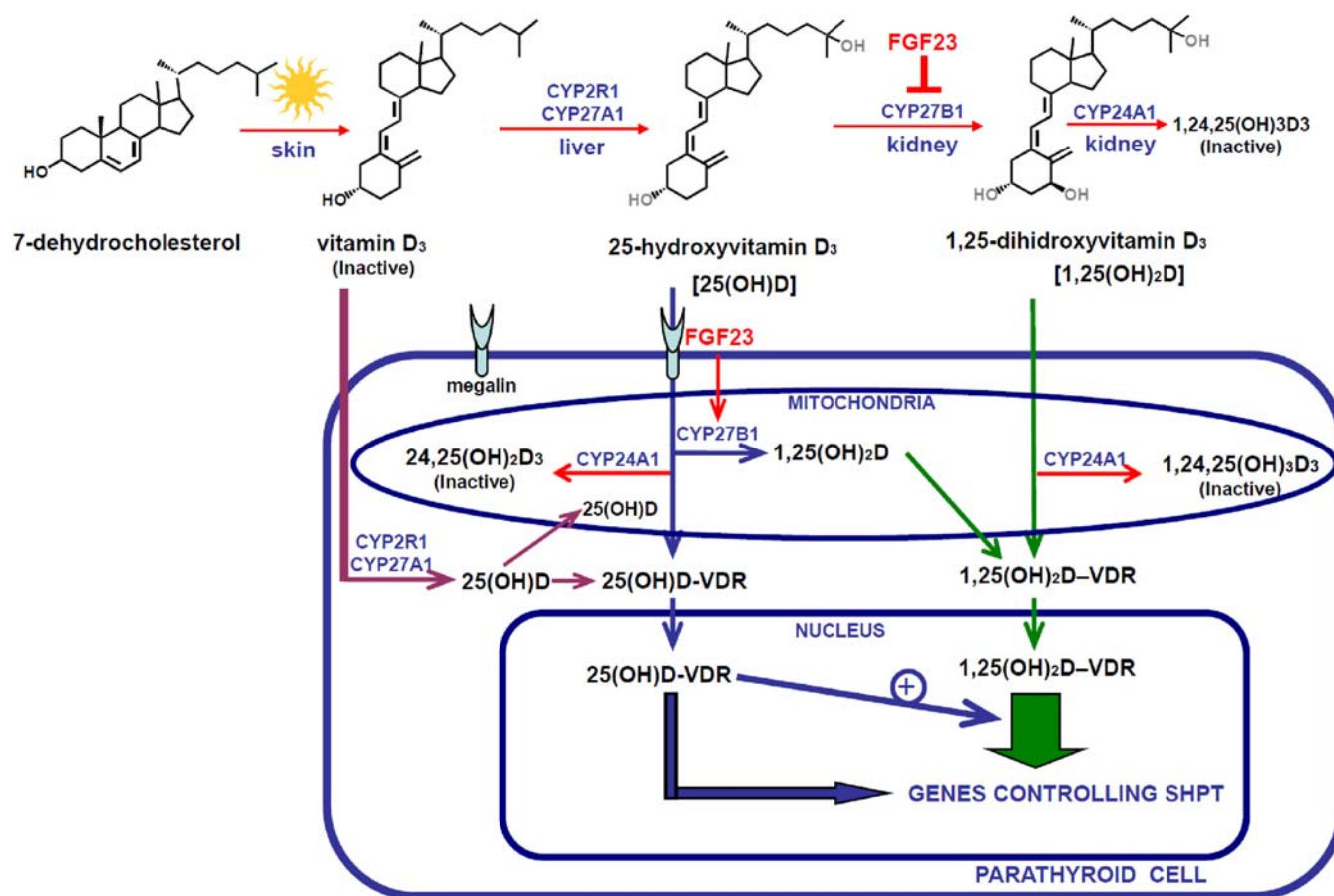
The following section updates our understanding of (1) the pathogenic mechanisms impairing the ability of the injured kidney to maintain serum 25(OH)D levels and appropriate renal or extra-renal 1,25(OH)<sub>2</sub>D production and (2) the adverse impact of the abnormalities in renal and extra-renal vitamin D metabolism in response to vitamin D supplementation, 25(OH)D administration or 1,25(OH)<sub>2</sub>D (analogs) replacement therapy. This information is essential to resolve current controversies in the appropriate management of vitamin D and 1,25(OH)<sub>2</sub>D deficiency in CKD to improve renal and cardiovascular outcomes.

### 3. Alterations in vitamin D bioactivation to 25(OH)D in chronic kidney disease

Vitamin D is not a vitamin, as “vitamin” is the name for compounds that our body cannot produce and, in mammals, vitamin D is produced by the skin (see Chapter 3). As summarized in Fig. 79.2 [18], skin exposure to sunlight of the ultraviolet B range (UV-B) converts 7-dehydrocholesterol into vitamin D<sub>3</sub> (cholecalciferol) [18], a conversion attenuated by sun blockers [30]. CKD impairs this photo-activation of the vitamin D precursor by the skin [31]. Large genome-wide association studies (GWASs) in white Europeans have identified polymorphisms in 7-dehydrocholesterol reductase (DHCR7, the enzyme that converts 7-dehydrocholesterol into cholesterol) that are associated with reduced vitamin D levels [32], likely due to DHCR7 removal of the substrate for vitamin D synthesis. In a European cohort of 500 CKD children, vitamin D deficiency was prevalent in two-thirds of the cohort [33] but was not independently associated with these polymorphisms. However, the abnormal cholesterol metabolism in CKD suggests that skin DHCR7 may play a larger role in the regulation of vitamin D status than previously recognized.

Vitamin D<sub>3</sub> and its homolog in plants, vitamin D<sub>2</sub> (ergocalciferol), are inactive prohormones, which need to undergo a two-step bio-activation to the potent calcitropic steroid hormone 1,25(OH)<sub>2</sub>D<sub>2</sub> or D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) to exert their biological actions (see Chapter 4). In the first activation step, vitamin D<sub>3</sub> undergoes 25-hydroxylation mainly in the liver by two cytochromes





**FIGURE 79.2 Systemic and parathyroid vitamin D metabolism and actions.** Two hydroxylation steps, 25-hydroxylation in the liver and  $1\alpha$ -hydroxylation in the kidney, convert inactive vitamin D produced in the skin by exposure to UV light, into the potent calcitropic hormone  $1,25(\text{OH})_2\text{D}$ . The kidney tightly controls serum  $1,25(\text{OH})_2\text{D}$  by inducing its degradation by CYP24A1 when serum levels raise above normal. Parathyroid cells express all the cytochromes P450 required for  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  production and for their metabolic inactivation. The cells also express megalin, an endocytosis receptor required for the cellular uptake of  $25(\text{OH})\text{D}$ , which circulates bound to its carrier, DBP. Once inside the parathyroid cell, the degree of  $1,25(\text{OH})_2\text{D}$ -VDR complex formation, required to induce the transcription of the genes that control secondary hyperparathyroidism (SHPT), will depend on the net balance between stimuli for  $1,25(\text{OH})_2\text{D}$  activation versus degradation. Note the opposite regulation by FGF23 of  $1,25(\text{OH})_2\text{D}$  production by CYP27B1 in the kidney and in parathyroid cells.  $1,25(\text{OH})_2\text{D}$  is the most potent inducer of VDR activation (the widest arrow) and transcriptional regulation of vitamin D-responsive genes. However,  $25(\text{OH})\text{D}$  also binds and activates the VDR with a 100–200 lower potency (a narrower arrow). Importantly,  $25(\text{OH})\text{D}$  can synergize with  $1,25(\text{OH})_2\text{D}$  activation of the VDR (+). An autocrine/paracrine induction of the  $25(\text{OH})\text{D}$ – $1,25(\text{OH})_2\text{D}$  synergy for VDR activation should maximize the biological response to VDR activation with a minimal risk of hypercalcemia or hyperphosphatemia. FGF23, fibroblast growth factor 23; VDR, vitamin D receptor.

P450s, mitochondrial CYP27A1, and microsomal CYP2R1. CYP27A1, an enzyme whose inactivating mutation causes abnormal cholesterol and bile metabolism, but not rickets, cannot hydroxylate vitamin  $\text{D}_2$  [34]. In humans, CYP2R1 appears to be the most critical vitamin D-25-hydroxylase because mutations in this gene but not the CYP27A1 gene results in severe vitamin D deficiency (serum  $25(\text{OH})\text{D}$  levels below 10 ng/mL) [35] (see Chapter 67). Interestingly, simultaneous ablation of the CYP27A1 and CYP2R1 genes only reduced circulating  $25(\text{OH})\text{D}$  levels by 50%, thus suggesting an important contribution of other 25-hydroxylases [36]. CYP3A4, a major drug-metabolizing enzyme mainly located in the liver and intestine, also has 25-

hydroxylating activity but prefers  $1\alpha$ -hydroxylated substrates [37]. In addition to the liver, skin and parathyroid cells, as well as monocyte/macrophages, express 25-hydroxylase activity, but the impact on cellular function or on serum  $25(\text{OH})\text{D}$  levels has not been explored [38–40].

The vitamin D status of an individual is estimated by measurements of serum  $25(\text{OH})\text{D}$  because serum  $25(\text{OH})\text{D}$  levels accurately reflect the efficacy of hepatic 25-hydroxylases to convert most circulating vitamin D into  $25(\text{OH})\text{D}$  [41]. Also, the 15 to 18 day biological half-life of  $25(\text{OH})\text{D}$  prevents an underestimation of vitamin D status due to rapid fluctuations of serum  $25(\text{OH})\text{D}$  levels. Currently, only mass spectrometry

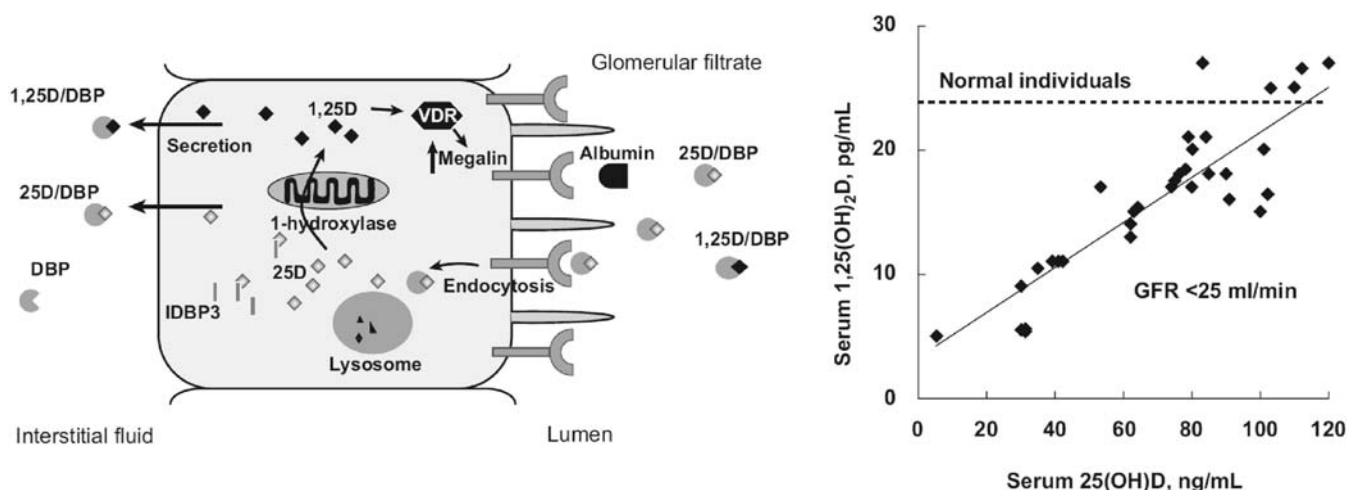
accurately measures 25(OH)D levels (see Chapter 50 [Vol. 1 of this book]). Chapter 49 (Vol. 1 of this book) discusses the accuracy and specificity of available assays.

CKD-induced changes in the expression or activity of the hepatic or extra-hepatic 25-hydroxylase may contribute to the high variability in the response of these patients to ergocalciferol or cholecalciferol supplementation. However, the high degree of vitamin D deficiency in CKD is not caused by a defective conversion of vitamin D into 25-hydroxyvitamin D but mainly by progressive reductions in renal megalin. Severe vitamin D deficiency was noted in megalin-null mice, uncovering a previously unrecognized role for a normal kidney in the maintenance of normal serum 25(OH)D [42]. This role involves the uptake of 25(OH)D from the urinary ultra-filtrate and its recycling back to the circulation, summarized in Fig. 79.3. Briefly, 25(OH)D, a lipid soluble molecule, circulates in the blood mostly bound to its carrier, the vitamin D-binding protein (DBP), with a molecular weight similar to that of albumin, another carrier of vitamin D metabolites with lower binding affinity than DBP. The 25(OH)D/DBP or 25(OH)D/albumin complex is filtered through the glomerulus and reenters proximal tubular cells through a process of endocytosis that requires adequate tubular levels of the endocytosis receptor megalin, a member of the low-density lipoprotein (LDL) receptor superfamily. Chapter 7 provides a more detailed discussion of DBP.

In CKD, an early loss of renal megalin is evident in rats after only 2 weeks of the induction of renal injury [43]. Thus, defective renal 25(OH)D uptake provides a mechanistic explanation for the high incidence of vitamin D deficiency at all CKD stages [9]. Supplementation with ergocalciferol, cholecalciferol, or 25(OH)D increases the proportion of DBP carrying 25(OH)D in the blood and the amount of filtered 25(OH)D/DBP complex available for megalin-mediated endocytosis, regardless of a normal or reduced glomerular filtration rate (GFR). Undoubtedly, in CKD, progressive reductions in megalin and in GFR contribute to the difficulties in normalizing serum 25(OH)D levels in response to vitamin D supplementation [44,45].

Because vitamin D induces the expression of renal megalin [46], the correction of vitamin D deficiency at early CKD stages could attenuate renal megalin loss. In predialysis CKD patients, urinary megalin excretion correlates directly with urinary protein and inversely with circulating 25(OH)D levels. Thus, measurements of urinary megalin in CKD could provide an early biomarker of the degree of renal proximal tubular cell injury [47] to help personalize vitamin D supplementation strategies.

The question remains as to what is the optimal serum 25(OH)D level or vitamin D supplementation regimen in CKD? A Japanese study in a large population of predialysis patients with CKD3–5, demonstrated that serum levels of 25(OH)D above 23 ng/mL significantly



**FIGURE 79.3** Defective renal 25(OH)D uptake limits renal calcitriol synthesis in CKD. Left panel: Circulating 25(OH)D (25D) bound to its carrier vitamin D-binding protein (DBP) is filtered by the kidney and internalized into proximal tubular cells via megalin-mediated endocytosis. On its release from DBP, 25(OH)D is either delivered to  $1\alpha$ -hydroxylase by intracellular vitamin D-binding protein 3 (IDBP3) for its bioactivation to 1,25(OH)<sub>2</sub>D (1,25D), or it reenters the circulation. Calcitriol induces renal megalin expression, thereby generating a cycle that ensures normal systemic 25(OH)D and calcitriol levels as well as the reabsorption of low-molecular-weight proteins from the glomerular filtrate, including albumin. In CKD, decreases in GFR and low megalin content contribute to impair 25(OH)D uptake and protein reabsorption. Right panel: The strong correlation between serum levels of 25(OH)D and calcitriol [1,25(OH)<sub>2</sub>D] in hemodialysis patients (GFR < 25 mL/min), which is absent in normal individuals (dotted line), demonstrates impaired 25(OH)D availability for its bioactivation to calcitriol by the remnant renal  $1\alpha$ -hydroxylase in CKD. Only supraphysiological concentrations of 25(OH)D normalize serum calcitriol levels. CKD, chronic kidney disease; VDR, vitamin D receptor. Adapted from Ref. [89].

reduced the risk of CKD progression [11]. Notably, this 25(OH)D concentration was shown insufficient to reduce PTH in adult CKD. In children with CKD2–4, supplementation with ergocalciferol (2000 units IU daily) was sufficient to delay the onset of SHPT [48] and to lower serum PTH. A recent meta-analysis of four-randomized controlled trials in pre-dialysis and hemodialysis patients supported these findings [49]. However, in adult patients with CKD stages 3–4, daily administration of 4000 IU of cholecalciferol for 1 month followed by 2000 IU daily for 2 additional months increased serum 25(OH)D levels from 14 to 37 ng/mL without reducing PTH [44]. In a similar cohort of patients, ergocalciferol administration (50,000 IU, every 2 weeks) effectively reduced serum PTH only in 50% of the patients achieving serum 25(OH)D levels above 35 ng/mL [50]. Instead, daily doses of 8000 IU of cholecalciferol for 12 weeks significantly reduced PTH without hypercalcemia or hyperphosphatemia [51], suggesting that high daily doses of cholecalciferol may represent a superior supplementation regimen. While the optimal vitamin D replacement regimen in individuals with CKD remains unclear, it is well recognized that vitamin D supplementation regimens will depend on the CKD stage and should be personalized to improve outcomes rather than to normalize serum 25(OH)D levels. Significantly, a typical U-shaped curve for the association between serum 25(OH)D and relative risk of mortality in healthy women [52] underscores the importance of avoiding an excess of serum 25(OH)D. Therefore, a better understanding of CKD-induced abnormalities of renal and extra-renal 1,25(OH)<sub>2</sub>D synthesis and of 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism is required (to be discussed in the following).

#### 4. Alterations in renal 1,25(OH)<sub>2</sub>D synthesis and 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism in chronic kidney disease

The 1 $\alpha$ -hydroxylation of 25(OH)D to produce 1,25(OH)<sub>2</sub>D, the most active endogenous vitamin D metabolite, is the most critical and tightly regulated of the two steps in vitamin D bio-activation. This step is catalyzed by the mitochondrial cytochrome P450 mixed-function oxidase CYP27B1 or 1 $\alpha$ -hydroxylase [19] and occurs mainly, but not exclusively, in the renal proximal convoluted tubules (see Chapter 8 [Vol. 1 of this book]). Indeed, the kidney is the main, if not the only, contributor to circulating 1,25(OH)<sub>2</sub>D, at least under physiological conditions. Therefore, for more than 3 decades, nephrologists have used oral or intravenous 1,25(OH)<sub>2</sub>D/analogues to correct the progressive reductions in renal 1,25(OH)<sub>2</sub>D production when GFRs fall below 50% of normal [53–56].

Serum 1,25(OH)<sub>2</sub>D levels should be maintained within the narrow limits that ensure the functional integrity of the calcium/1,25(OH)<sub>2</sub>D/PTH and phosphorus/1,25(OH)<sub>2</sub>D/FGF23–klotho axes controlling bone and mineral homeostasis with minimal risk of renal or cardiovascular injury due to hypercalcemia or hyperphosphatemia. Therefore, renal 1,25(OH)<sub>2</sub>D production is strictly controlled through a tight and coordinated regulation of 1,25(OH)<sub>2</sub>D synthesis by 1 $\alpha$ -hydroxylase and of 1,25(OH)<sub>2</sub>D catabolism by CYP24A1 or 24-hydroxylase, the cytochrome P450 that catalyzes 25(OH)D and 1,25(OH)<sub>2</sub>D metabolic inactivation [18]. Indeed, in normal individuals, when 1,25(OH)<sub>2</sub>D production rate increases above normal levels, the metabolic clearance rate of 1,25(OH)<sub>2</sub>D is accelerated [57]. Accordingly, severe hypercalcemia and nephrocalcinosis develop in children and adults with a loss-of-function mutation of the 24-hydroxylase gene [58,59] (see Chapter 69). These pathological features mimic the phenotype of high serum 1,25(OH)<sub>2</sub>D, severe hypercalcemia, hyperphosphatemia, osteoporosis, nephrocalcinosis, and vascular calcification in the 24-hydroxylase null mouse [60].

1 $\alpha$ -hydroxylase and 24-hydroxylase expression and activity are tightly regulated by the three mineralotropic hormones PTH, FGF23, and 1,25(OH)<sub>2</sub>D and also by the main modulators of their circulating levels, serum calcium, and phosphate. PTH, hypocalcemia, and hypophosphatemia are the major CYP27B1 inducers, while FGF23, klotho, hyperphosphatemia, hypercalcemia, and 1,25(OH)<sub>2</sub>D are the main repressors. Conversely, CYP24A1 is induced by 1,25(OH)<sub>2</sub>D and FGF23 and inhibited by PTH (reviewed in Ref. [19]).

In health, PTH is the most potent stimulator of renal 1,25(OH)<sub>2</sub>D synthesis (see Chapter 8). Patients with hypoparathyroidism have low 1,25(OH)<sub>2</sub>D levels despite persistent hypocalcemia [61] and parathyroidectomy severely blunt the induction of renal 1 $\alpha$ -hydroxylase by hypocalcemia [62]. Dietary phosphate restriction also increases renal 1,25(OH)<sub>2</sub>D production, serum 1,25(OH)<sub>2</sub>D, and mRNA levels of renal 1 $\alpha$ -hydroxylase [63,64] despite decreases in serum PTH [65]. Growth hormone may play a role in low phosphorus-driven induction of renal-1 $\alpha$ -hydroxylase mRNA levels and serum 1,25(OH)<sub>2</sub>D, as hypophysectomy blocks, whereas injection of growth hormone or IGF1 to hypophysectomized rats restores renal 1,25(OH)<sub>2</sub>D production [66].

FGF23 and klotho strongly suppress renal CYP27B1 mRNA levels and 1,25(OH)<sub>2</sub>D synthesis, as conclusively demonstrated by the high basal CYP27B1 mRNA levels and high serum 1,25(OH)<sub>2</sub>D in the FGF23 and klotho-null mice despite severe hypercalcemia, hyperphosphatemia, and low PTH [67] and also despite the high FGF23 in the klotho-null mice [68] (see Chapter 19). The latter supports either a key role for the FGF23/membrane or



soluble klotho complex to suppress 1 $\alpha$ -hydroxylase in an intact kidney, as suggested by the proximal tubule specific klotho ablation [69], or an FGF23-independent suppression of 1 $\alpha$ -hydroxylase by  $\alpha$ -klotho.

1,25(OH)<sub>2</sub>D feedback inhibits 1 $\alpha$ -hydroxylase activity [70,71] and gene expression [72–75], the latter not through classical VDR-trans-repression but through the suppression of the PTH/cAMP induction of 1 $\alpha$ -hydroxylase gene expression [76], and induces renal CYP24A1 gene transcription through VDR binding to the two proximal VDREs on the CYP24A1 gene promoter [77]. Several indirect mechanisms also contribute to 1,25(OH)<sub>2</sub>D inhibition of net renal 1,25(OH)<sub>2</sub>D production in vivo, including 1,25(OH)<sub>2</sub>D-mediated increases in serum calcium and phosphorus levels, suppression of serum PTH, and induction of FGF23 [12,21] and renal klotho [67], all of which also induce 24-hydroxylase.

The most critical advances in our understanding of the coordinated regulation of renal 1,25(OH)<sub>2</sub>D synthesis and catabolism by PTH, FGF23, and 1,25(OH)<sub>2</sub>D in health came from very recent work from the Pike laboratory reviewed in Ref. [78]. Using complex genomic approaches and loss-of-function studies, they identified CYP27B1 gene regulatory modules that are highly specific for renal proximal tubular cells and the sole determinants of 1 $\alpha$ -hydroxylase responses to PTH, FGF23, and 1,25(OH)<sub>2</sub>D [25]. These four submodules are dispersed and located within specific introns in the adjacent *Mettl1* and *Mettl21b* genes. While the submodules in *Mettl1* control PTH induction of the 1-hydroxylase gene, three separate submodules in the *Mettl21b* gene mediate FGF23 inhibition, and all four modules are involved in its suppression by 1,25(OH)<sub>2</sub>D [25].

The high translational relevance of these novel CYP27B1 regulatory modules resides in the demonstration that specific deletion of the PTH-responsive module in mice is sufficient to cause a 99% reduction in 1 $\alpha$ -hydroxylase gene expression and a bone and mineral phenotype similar to that of the *Cyp27b1* null mice, with marked decreases in circulating 1,25(OH)<sub>2</sub>D (absent in the *Cyp27b1* null mice) and in intestinal calcium absorption, leading to elevations in circulating PTH, reductions in FGF23, and renal *Cyp24a1* gene expression, as well as decreased bone mass. Proof of the coordinated renal regulation of *Cyp27b1* and *Cyp24a1* was found when rescuing these mouse models with high-calcium and high-phosphorus diets, which raised serum calcium, phosphate, and FGF23 levels; suppressed PTH; and normalized *Cyp24a1* expression, resulting in undetectable 1,25(OH)<sub>2</sub>D levels. As these modules are not present in non-renal cells expressing 1 $\alpha$ -hydroxylase, these findings also ruled out the potential contribution of non-renal sources of 1,25(OH)<sub>2</sub>D to the very low but detectable circulating levels of 1,25(OH)<sub>2</sub>D upon

ablation of the 1 $\alpha$ -hydroxylase regulating modules, at least in mice with normal kidneys (see Chapter 8).

In addition, Pike and collaborators have identified another exclusive renal module, which mediates the opposing regulation of *Cyp24a1* by PTH and FGF23, and which is associated with the disappearance of remnant 1,25(OH)<sub>2</sub>D upon dietary normalization of PTH and FGF23 in mice with an ablated PTH-responsive module for *Cyp27b1*. It is comprised of one segment located inter-genically downstream from the *Cyp24a1* transcriptional start site. Importantly, mice with exclusive deletion of the PTH responsive module controlling *Cyp27b1* expression also had higher serum 25(OH)D than wild-type mice, which normalized after feeding with the high-calcium, high-phosphorus rescue diet that induced FGF23 and *Cyp24a1* expression. This corroborates the importance of the coordinated regulation of 1 $\alpha$ -hydroxylase and 24-hydroxylase expression and activity by PTH and FGF23 by a normal kidney to prevent elevations in circulating 25(OH)D above normal [78]. Significantly, a second module that controls *Cyp24a1* transactivation by 1,25(OH)<sub>2</sub>D was identified exclusively in non-renal VDR-responsive cells but not in renal cells [78]. This underscores the distinct transactivation of the renal-constitutively expressed and non-renal-inducible *Cyp24a1* by 1,25(OH)<sub>2</sub>D.

At present, it is unknown at what point in the course of CKD progression, this highly inter-coordinated transcriptional regulation of *Cyp27b1* and *Cyp24a1* expression by 1,25(OH)<sub>2</sub>D, PTH, and FGF23 is lost. CKD also impairs the response to the classical regulators of renal 1,25(OH)<sub>2</sub>D synthesis. The accumulation of N-terminally truncated PTH fragments or C-terminal PTH, known to directly inhibit renal 1 $\alpha$ -hydroxylase [79], could partly account for the reduced 1,25(OH)<sub>2</sub>D production in response to either calcium restriction [80] or high serum PTH [81]. Phosphorus restriction in patients with end-stage renal disease [82] or in severely uremic dogs [83] failed to increase serum 1,25(OH)<sub>2</sub>D. The accumulation of uremic toxins also reduces renal 1,25(OH)<sub>2</sub>D synthesis, as shown by the infusion of uremic plasma ultra-filtrate into normal rats and the potent inhibition of 1 $\alpha$ -hydroxylase activity in vitro by low-molecular-weight compounds from uremic plasma ultra-filtrate [84]. Accordingly, in partially nephrectomized rats, increases in dietary protein intake that induce the production of uremic toxins markedly suppress 1,25(OH)<sub>2</sub>D synthesis.

Significantly, the abnormal regulation of 1 $\alpha$ -hydroxylase expression and activity in proximal tubules in CKD is not the only determinant of the progressive decreases in circulating 1,25(OH)<sub>2</sub>D levels. In fact, in early studies in hemodialysis patients with a GFR below 25 mL/min, accompanied by severely limited renal-1 $\alpha$ -hydroxylase activity and likely very high serum FGF23, serum



1,25(OH)<sub>2</sub>D levels were low only if serum 25(OH)D concentrations were low or normal [85]. Importantly, serum levels of 1,25(OH)<sub>2</sub>D could be normalized in these patients simply by increasing the serum concentrations of 25(OH)D to 100–200 ng/mL through oral supplementation. Furthermore, a strong correlation exists between serum levels of 25(OH)D and 1,25(OH)<sub>2</sub>D that does not occur in individuals with normal kidney function (see Fig. 79.3, right panel). A more recent prospective study corroborated the progressive increases in 1,25(OH)<sub>2</sub>D in vitamin D–deficient hemodialysis patients treated with 25(OH)D for 6 months [86]. Kinetic studies in dogs with moderate or severe uremia demonstrated that the increases in serum 1,25(OH)<sub>2</sub>D in response to 25(OH)D supplementation involved increases in production rates [87], thus supporting the importance of the maintenance of serum 25(OH)D levels for 1,25(OH)<sub>2</sub>D production by the remnant renal enzyme even in end-stage renal disease.

Even though the strong correlation between serum 25(OH)D and 1,25(OH)<sub>2</sub>D may result from impaired 25(OH)D availability for renal 1 $\alpha$ -hydroxylase described before [85] and caused partially by megalin reductions [43], it is also possible that the high serum 25(OH)D competes with the low 1,25(OH)<sub>2</sub>D as the substrate for Cyp24a1, thereby increasing serum 1,25(OH)<sub>2</sub>D by reducing its catabolism. However, the contribution of extra-renal 1,25(OH)<sub>2</sub>D production to the observed elevations in serum 1,25(OH)<sub>2</sub>D after 25(OH)D administration in advanced CKD should not be ruled out, as will be discussed in the following.

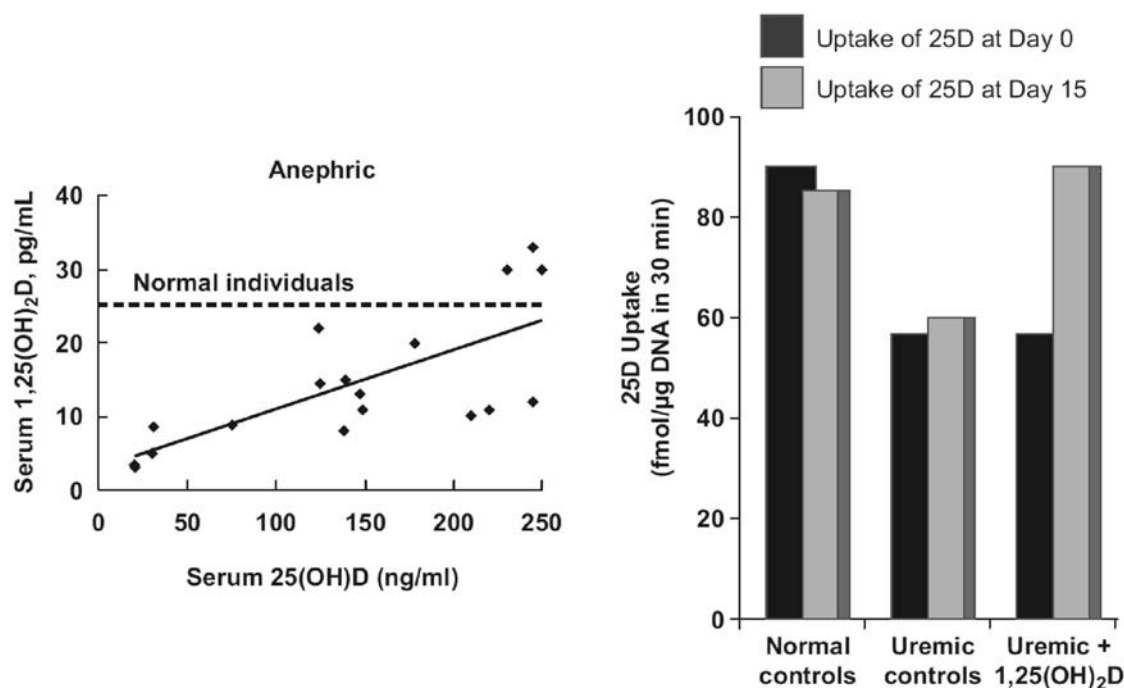
CKD may also impair substrate availability for 25(OH)D catabolism. In experimental and human CKD, despite marked elevations in renal 24-hydroxylase mRNA levels induced by the high FGF23, serum levels of 24,25-dihydroxyvitamin D, a biomarker of Cyp24a1 activity, are low rather than elevated [88,89]. These findings have corroborated the early work by Ishimura and colleagues in rats overexpressing renal Cyp24a1 [90]. More importantly, low serum 24,25-dihydroxyvitamin D levels in these transgenic rats were not caused by accelerated degradation of vitamin D metabolites. Instead, an impaired renal uptake of 25(OH)D due to albuminuria prevented its degradation by the overexpressed 24-hydroxylase [90]. Therefore, CKD impairs the response to FGF23 induction of 24-hydroxylase to avoid vitamin D toxicity, possibly through the same reductions in renal megalin compromising 25(OH)D uptake for 1,25(OH)<sub>2</sub>D synthesis. A recent prospective trial on ergocalciferol supplementation at earlier CKD stages corroborated a reduction in vitamin D catabolism, as measured by 24,25(OH)<sub>2</sub>D/25(OH)D ratios. This reduction occurred despite unchanged serum PTH, FGF23, DBP, or albumin levels and was attributed to defective substrate availability

[91]. Taken together, these findings indicate that progressive impairments in 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism in the course of CKD render these patients more sensitive to vitamin D toxicity than previously estimated. Undoubtedly, to comply with current KDIGO guidelines on vitamin D interventions, serial measurements of changes in vitamin D metabolite ratios (1,25(OH)<sub>2</sub>D/25(OH)D; 24,25(OH)<sub>2</sub>D/25(OH)D, and 1,25(OH)<sub>2</sub>D/24,25(OH)<sub>2</sub>D) with CKD progression would better reflect the degree of impaired control of renal 1,25(OH)<sub>2</sub>D synthesis and of 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism in response to elevations in PTH and FGF23 than single measurements of serum 25(OH)D and 1,25(OH)<sub>2</sub>D levels.

### 5. Alterations in 1,25(OH)<sub>2</sub>D production by extrarenal 1 $\alpha$ -Hydroxylases in chronic kidney disease

In CKD, non-renal 1 $\alpha$ -hydroxylases contribute to systemic 1,25(OH)<sub>2</sub>D levels, as conclusively demonstrated in bilaterally nephrectomized patients undergoing hemodialysis [92]. Furthermore, there is a strong correlation between serum levels of 25(OH)D and 1,25(OH)<sub>2</sub>D depicted in Fig. 79.4 (left panel) suggesting that CKD also impairs 25(OH)D availability to non-renal 1 $\alpha$ -hydroxylases. In fact, in the absence of renal mass, the very low serum 1,25(OH)<sub>2</sub>D levels can be raised to normal levels with appropriate 25(OH)D supplementation [92]. This early work also supports the contribution of extra-renal 1,25(OH)<sub>2</sub>D synthesis to the increases in serum 1,25(OH)<sub>2</sub>D observed in hemodialysis patients on 25(OH)D [92] or cholecalciferol supplementation [93].

The impact of CKD on 1,25(OH)<sub>2</sub>D production by non-renal 1 $\alpha$ -hydroxylases was first examined in monocyte/macrophages, as the enzyme is identical to the renal 1 $\alpha$ -hydroxylase though more readily accessible. Surprisingly, monocytes derived from peripheral blood mononuclear cells from hemodialysis patients had a higher expression of 1 $\alpha$ -hydroxylase than monocytes from normal individuals [94]. This suggests a role for the 1 $\alpha$ -hydroxylase of monocytes in compensating for the defective 1,25(OH)<sub>2</sub>D production by the proximal tubule cells of an injured kidney. The higher monocyte 1 $\alpha$ -hydroxylase activity appears to reflect a defective feedback inhibition of the enzyme expression/activity by the low serum 1,25(OH)<sub>2</sub>D levels, as monocytes lack PTH receptors, and monocyte 1 $\alpha$ -hydroxylase does not respond to changes in serum calcium and phosphorus levels as the renal enzyme does [94]. However, monocyte 1 $\alpha$ -hydroxylase is also inhibited by high serum FGF23 [95]. In fact, the V<sub>max</sub> of monocyte 1 $\alpha$ -hydroxylase, a measure of the cellular content of the



**FIGURE 79.4** 1,25(OH)<sub>2</sub>D corrects the impaired 25(OH)D availability to nonrenal 1 $\alpha$ -hydroxylase in CKD. Left panel: The strong correlation between serum levels of 25(OH)D and 1,25(OH)<sub>2</sub>D in bilaterally nephrectomized patients (anephrics) demonstrates impaired 25(OH)D availability for its bioactivation to 1,25(OH)<sub>2</sub>D by nonrenal 1 $\alpha$ -hydroxylase. Only supraphysiological concentrations of 25(OH)D normalize serum 1,25(OH)<sub>2</sub>D levels. Right panel: 25(OH)D uptake (30 min) at day 0 (left bar) and at day 15 (right bar) by peripheral blood monocytes from normal individuals (normal) and hemodialysis patients (uremic) receiving either vehicle (controls) or intravenous 1,25(OH)<sub>2</sub>D three times a week for 15 days. CKD, chronic kidney disease. Left panel: Adapted from Ref. [92]. Right panel: Modified from Ref. [96].

enzyme, correlates inversely with serum 1,25(OH)<sub>2</sub>D and is exquisitely sensitive to downregulation by physiological levels of 1,25(OH)<sub>2</sub>D [96].

The tight control of monocyte–macrophage 1,25(OH)<sub>2</sub>D synthesis by physiological concentrations of serum 1,25(OH)<sub>2</sub>D in CKD patients differs markedly with that reported in monocytes from patients with sarcoidosis and other granulomatous diseases. In these disorders, hypercalcemic episodes result from the inability of the high serum 1,25(OH)<sub>2</sub>D concentrations to either suppress 1,25(OH)<sub>2</sub>D synthesis by the disease-activated macrophages or to induce 24-hydroxylase [97].

The regulation of CYP27B1 in non-renal cells differs markedly from the response to the same modulators in renal tubular cells, as they lack the regulatory modules that mediate the renal responses to PTH, FGF23, and 1,25(OH)<sub>2</sub>D. Furthermore, there is a distinct and cell-specific response to the same modulator. For instance, the high circulating levels of FGF23, common in CKD patients, inhibit monocyte/macrophage 1,25(OH)<sub>2</sub>D production [95], but induce 1 $\alpha$ -hydroxylase mRNA levels in cells from normal parathyroid glands [98]. The distinct regulation of renal and parathyroid 1 $\alpha$ -hydroxylase by FGF23 and that of monocyte/macrophage 1 $\alpha$ -hydroxylase in patients with CKD compared with individuals with inflammatory disorders underscores the

complexity behind the design of effective vitamin D interventions that improve clinical outcomes by simultaneously correcting endocrine and autocrine/paracrine VDR actions in the most relevant target cells/tissues.

Interestingly, CKD also impairs monocyte 25(OH)D uptake [96], as shown in peripheral blood monocytes from hemodialysis patients with a 40% lower uptake of 25(OH)D compared with monocytes from normal individuals (Fig. 79.4, right panel) [96]. This defective uptake can be corrected by normalizing the low-serum 1,25(OH)<sub>2</sub>D levels of hemodialysis patients through intravenous 1,25(OH)<sub>2</sub>D administration for 15 days (Fig. 79.4, right panel) [96]. Monocytes do not express megalin; however, the time course for 25(OH)D uptake is not linear, as would be expected for the diffusion of a free lipophilic compound through the plasma membrane [96]. Since 1,25(OH)<sub>2</sub>D modulates LDL receptor expression and function in the human monocytic cell line HL60 [99,100], and megalin is a member of the LDL receptor superfamily, a similar LDL receptor regulation may partially account for 1,25(OH)<sub>2</sub>D correction of the impaired uptake of 25(OH)D by peripheral blood monocytes from hemodialysis patients.

In the parathyroid gland, a defective uptake of 25(OH)D by parathyroid cells due to megalin reductions [101] could aggravate the adverse impact of vitamin D

deficiency for the correction of PTH synthesis by locally produced  $1,25(\text{OH})_2\text{D}$  [102]. Distinct levels of parathyroid megalin may contribute to the high variability demonstrated for CKD patients' suppression of PTH in response to vitamin D supplementation despite normalization of serum  $25(\text{OH})\text{D}$ .

The benefits of local  $1,25(\text{OH})_2\text{D}$  production extend beyond the control of SHPT. In a cohort of 158 hemodialysis patients, cholecalciferol supplementation resulted in higher serum  $25(\text{OH})\text{D}$  levels and also in elevations in  $1,25(\text{OH})_2\text{D}$  and albumin. More importantly, cholecalciferol supplementation reduced serum calcium, phosphorus, PTH, brain natriuretic peptide, left ventricular mass index, and the dose requirement for erythropoietin stimulating agents and for  $1,25(\text{OH})_2\text{D}$  (analogs) [93]. Importantly, in hemodialysis patients, with variable degrees of residual renal function, with poor or nonexistent megalin-mediated uptake of  $25(\text{OH})\text{D}$  from the urinary space, the capacity to increase serum  $25(\text{OH})\text{D}$  levels for local  $1,25(\text{OH})_2\text{D}$  synthesis decreases in direct proportion to their advanced proteinuria [103]. However, local  $25(\text{OH})\text{D}$  synthesis by parathyroid cells or monocytes could help normalize intracellular  $25(\text{OH})\text{D}$  levels for  $1,25(\text{OH})_2\text{D}$  synthesis and the local benefits VDR activation without changes in serum  $25(\text{OH})\text{D}$  levels.

Monocyte  $1,25(\text{OH})_2\text{D}$  production in response to cholecalciferol supplementation has also been reported to be beneficial in reducing the degree of systemic inflammation, measured by serum levels of the pro-inflammatory cytokines  $\text{TNF}\alpha$ , IL-8, and IL-6 [104]. In individuals with normal renal function, the capacity of monocytes to synthesize  $1,25(\text{OH})_2\text{D}$  from  $25(\text{OH})\text{D}$  to induce the gene expression of the antimicrobial peptide cathelicidin has provided a molecular explanation for the efficacy of a normal vitamin D status in protecting from tuberculosis [105] (see Chapter 94 and Chapter 98). Indeed, peripheral blood mononuclear cells from vitamin D-deficient African Americans restore their capacity to produce adequate amounts of cathelicidin following vitamin D supplementation. Undoubtedly, vitamin D-mediated improvement of the anti-inflammatory and antibacterial capacity of circulating monocytes in CKD patients has a beneficial impact on survival, as shown by the association of low levels of cathelicidin with a higher risk of death from infectious disease in dialysis patients [106].

The recent COVID 19 pandemic has provided additional evidence of the importance of a normal vitamin D status to effectively counteract the potent cytokine storm driving multi-organ damage to reduce disease severity and mortality rates in infected individuals [107–109]. It has also corroborated the high variability in outcomes in response to cholecalciferol or  $25(\text{OH})\text{D}$  dosage and timing (daily, weekly, monthly), baseline

vitamin D levels, and also racial differences. Notably, in the African American population, the risk of a clinically significant infection with normal circulating levels of  $25(\text{OH})\text{D}$  in the 30–40 ng/mL range is 2.45 higher than for levels above 40 ng/mL [110].

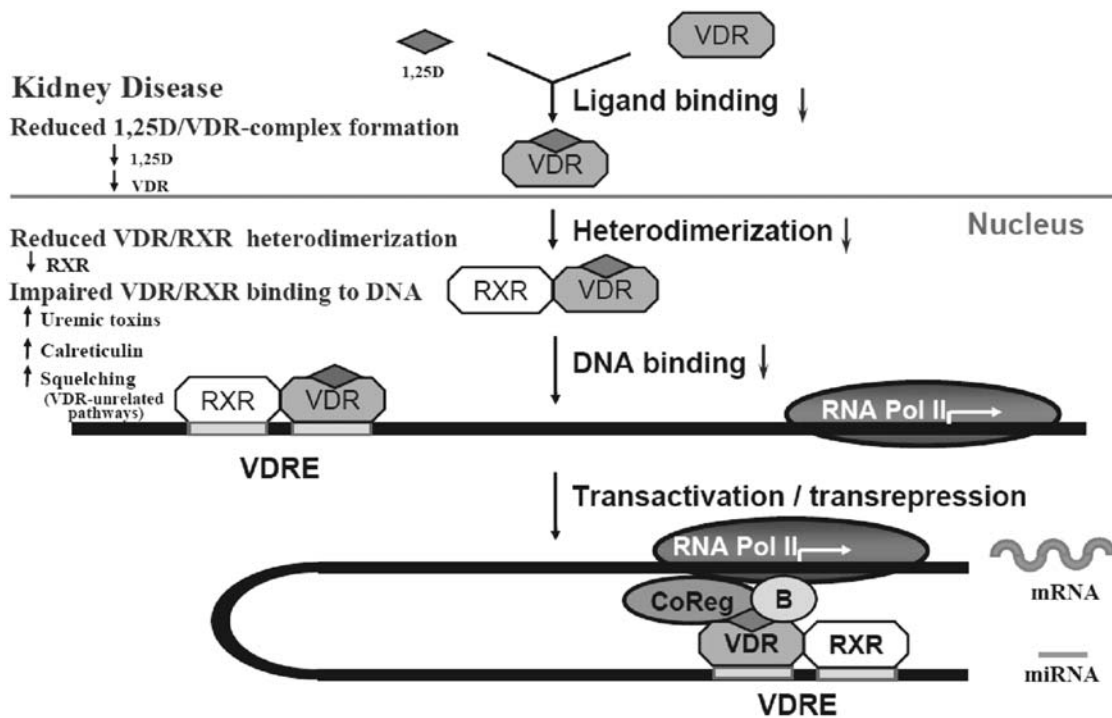
Although impaired uptake of circulating  $25(\text{OH})\text{D}$  in CKD patients may limit achieving full responses with vitamin D supplementation, it is certain that exclusive therapy with  $1,25(\text{OH})_2\text{D}$  (or analogs) will not correct vitamin D deficiency, thus fully preventing the reported benefits of autocrine/paracrine  $1,25(\text{OH})_2\text{D}$  production. Accordingly, current clinical guidelines recommend supplementation with nutritional vitamin D, mainly cholecalciferol, to correct vitamin D deficiency at all CKD stages before any intervention with  $1,25(\text{OH})_2\text{D}$  or its analogs (referred to as active vitamin D molecules) [111].

In summary, in the course of kidney disease, progressive reductions in serum  $25(\text{OH})\text{D}$  levels are key determinants of the severity of the defects in renal and extra-renal  $1,25(\text{OH})_2\text{D}$  synthesis causing low serum  $1,25(\text{OH})_2\text{D}$  and impaired  $1,25(\text{OH})_2\text{D}$  endocrine and autocrine/paracrine actions. Vitamin D supplementation can improve the impaired substrate availability for renal and non-renal  $1,25(\text{OH})_2\text{D}$  production by  $1\alpha$ -hydroxylases, normalize serum  $1,25(\text{OH})_2\text{D}$  levels and may also improve autocrine  $25(\text{OH})\text{D}$  synthesis.  $1,25(\text{OH})_2\text{D}$  replacement therapy can correct the reduced renal megalin content and the defective  $25(\text{OH})\text{D}$  uptake by non-renal cells but cannot correct  $25(\text{OH})\text{D}$  deficiency. Thus, the combination of nutritional vitamin D and  $1,25(\text{OH})_2\text{D}$  (or analogs) replacement therapy may simultaneously enhance  $25(\text{OH})\text{D}$  availability and improve renal and non-renal  $25(\text{OH})\text{D}$  uptake for endocrine and/or autocrine–paracrine actions of the  $1,25(\text{OH})_2\text{D}$ /VDR complex.

The assessment of changes in vitamin D metabolite ratios rather than single metabolite measurements could help personalize the design of safer and more effective vitamin D interventions. The identification of impaired catabolism of vitamin D metabolites in CKD calls for extreme caution to avoid vitamin D toxicity, particularly when using  $25(\text{OH})\text{D}$  to correct vitamin D deficiency or when simultaneously targeting vitamin D and  $1,25(\text{OH})_2\text{D}$  deficiency.

## 6. Alterations in $1,25(\text{OH})_2\text{D}$ /VDR endocrine and autocrine/paracrine actions in CKD

Most of  $1,25(\text{OH})_2\text{D}$ 's biological actions require its binding to the VDR, a member of the thyroid/retinoid nuclear receptor superfamily. The so-called “classical” genomic actions of the  $1,25(\text{OH})_2\text{D}$ /VDR complex (summarized in Fig. 79.5) involve ligand binding to the VDR,



**FIGURE 79.5** Abnormal VDR regulation of gene transcription in CKD. 1,25(OH)<sub>2</sub>D (1,25D)/VDR-transcriptional control of the expression of vitamin D-responsive genes involves ligand binding to VDR; VDR heterodimerization with RXR; DNA binding of the VDR/RXR complex, and recruitment of basal transcription factors (B), coregulator molecules (CoReg), and RNA polymerase II to activate or repress gene transcription, including genes coding for microRNA molecules. Kidney disease induces several mechanisms (listed on the left) responsible for impairing critical steps (indicated by blue arrows) in 1,25(OH)<sub>2</sub>D/VDR transcriptional activity. CKD, chronic kidney disease; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D-responsive element.

which induces a conformational change in the VDR molecule that facilitates heterodimerization with its major transcriptional partner, the retinoid X receptor (RXR), followed by the binding of the VDR/RXR complex to vitamin D-responsive sequences (VDREs) on the promoter regions of vitamin D-responsive genes to induce/repress their expression (see Chapters 10–13). Unliganded VDR can bind DNA and transcriptionally repress gene expression in a ligand-independent manner [111]. Among the different VDRE sequences, those consisting of two direct imperfect repeats with a spacer of three nucleotides (DR3) are associated with the highest affinity for the VDR. The ligand-activated VDR/RXR complex binds simultaneously to multiple VDREs within 100 kb either 5' or 3' from the transcription start site of a target gene rather than to a single VDRE, as demonstrated by conformation capture technology [112]. Distal and more proximal VDREs are juxtaposed by chromatin looping, thus facilitating the simultaneous recruitment of basic transcription factors, coactivator, and/or corepressor molecules at multiple VDR–RXR/VDRE complexes. The induction of the RANKL gene, essential in 1,25(OH)<sub>2</sub>D-driven osteoclastogenesis and bone resorption, is an example of the simultaneous occupation of multiple VDREs by the

VDR/RXR complex involved in its transactivation [113]. Similar single- or super-complexes of VDR/RXR bound to DNA transcriptionally activate/repress the expression of the 500–1000 genes that mediate the survival benefits of a normal vitamin D status, as will be specifically addressed for genes that influence CKD progression.

Furthermore, the suppression of CYP27B1 in renal proximal tubular cells by 1,25(OH)<sub>2</sub>D is another example that proximal regions in a gene promoter may play a role in transcriptional repression; however, unbiased genome-wide analysis has located four potent regulatory modules for the response to 1,25(OH)<sub>2</sub>D residing within specific introns in the adjacent genes *Mettl1* and *Mettl21b* [15,25].

A relevant developing field is 1,25(OH)<sub>2</sub>D/VDR transcriptional regulation of gene promoters that drive the expression of microRNAs (miRNAs) (Fig. 79.5) [114] (see Chapter 14). miRNAs are short (18–25 nucleotides) noncoding RNAs that bind the 3'-untranslated region of target mRNA decreasing either mRNA stability or protein translation, thereby controlling the expression of 30% of the genes in the genome. Highly relevant in CKD is 1,25(OH)<sub>2</sub>D (analog)-mediated upregulation of microRNA-145 in the vasculature [115] and the



induction of miR106b-5p secretion by circulating monocytes when macrophage VDR signaling is ablated [116]. While the former confers cardiovascular protection from calcium deposition, restenosis, and atherosclerosis, the latter has provided a causal link between immune cell activation by vitamin D deficiency and increased renin production by juxtaglomerular cells, as will be discussed in the following.

The  $1,25(\text{OH})_2\text{D}$ /VDR complex regulates gene transcription via several other mechanisms aside from direct repression or transactivation. The complex may induce genomic actions that involve an indirect control of the expression of an apparent target gene through the direct transcriptional control of an essential inducer or repressor. An example of such a mechanism is  $1,25(\text{OH})_2\text{D}$ -mediated suppression of ADAM17 gene expression to control parathyroid hyperplasia through the induction of C/EBP $\beta$  [117]. Alternatively, the cytosolic  $1,25(\text{OH})_2\text{D}$ /VDR complex may also bind main transcriptional regulators in signaling pathways unrelated to vitamin D biological actions that can drastically modify their transcriptional activity. An important example in CKD is the physical interaction of the VDR with  $\beta$ -catenin in the vasculature, which results in impaired Wnt activation to diminish vascular calcification [118] and the rapid progression of diabetic nephropathy to end-stage renal disease [119]. The  $1,25(\text{OH})_2\text{D}$ /VDR complex also affects the rates of mRNA translation, essential to maintain viability in cells forced to mount acute responses to counteract life-threatening conditions as starvation or strong growth signals. An important example of this process is vitamin D-mediated suppression of mammalian target of rapamycin (mTOR), as first demonstrated in breast cancer [120]. In CKD, this mechanism attenuates parathyroid gland enlargement [121] and VDR reductions, as will be discussed in the section on vitamin control of SHPT.

$1,25(\text{OH})_2\text{D}$  also exerts less well characterized rapid “nongenomic” actions, which occur within minutes of exposure to  $1,25(\text{OH})_2\text{D}$  and also involve the cytosolic VDR, although other potential receptors such as MARRS have been identified [122,123]. These rapid actions have the capacity to regulate intracellular calcium fluxes, as well as the degree of protein phosphorylation, acetylation, and subcellular localization to modify protein function and genomic signals (reviewed in Ref. [122]). The impact of these rapid vitamin D actions on pathways regulating the stability and/or processing of miRNAs and the intracellular trafficking by soluble klotho to improve survival is not known.

The intracellular levels of  $1,25(\text{OH})_2\text{D}$  and VDR determine the magnitude of  $1,25(\text{OH})_2\text{D}$ /VDR complex formation and, consequently, the efficacy for direct or indirect gene transactivation/trans-repression by the  $1,25\text{D}$ /VDR complex. Since both circulating

$1,25(\text{OH})_2\text{D}$  levels and local synthesis as well as cellular VDR content are reduced in CKD, VDR-mediated regulation of gene expression is greatly altered [19]. The vitamin D deficiency and  $1,25(\text{OH})_2\text{D}$  deficiency in CKD patients could also directly contribute to VDR reductions because  $1,25(\text{OH})_2\text{D}$  binding protects the VDR from proteosomal degradation [124]. Thus, when treating SHPT, the correction of vitamin D deficiency recommended by KDIGO guidelines could attenuate VDR reductions and, consequently, delay the escalation in  $1,25(\text{OH})_2\text{D}$  dosage required to compensate for lower parathyroid VDR levels, thereby ameliorating the risk of hypercalcemia, hyperphosphatemia, and propensity to calcifications.

In the course of CKD, reductions in cellular RXR content and accumulation of uremic toxins result in decreased  $1,25(\text{OH})_2\text{D}$ /VDR–RXR complex formation and the binding of the VDR/RXR complex to DNA, respectively, further decreasing the already defective response to vitamin D therapy caused by low VDR (Reviewed in Ref. [125]). Hypocalcemia associated with CKD increases nuclear levels of calreticulin, a protein that interacts with the DNA-binding domain of nuclear receptors including the VDR, further compromising  $1,25(\text{OH})_2\text{D}$ /VDR genomic actions [126]. The low serum  $1,25(\text{OH})_2\text{D}$  found in CKD could also affect the expression of essential coactivators or corepressor molecules [127] or the proper translational modifications required for their recruitment to the VDR transcription preinitiation complex. In fact, selective recruitment of corepressor molecules by  $1,25(\text{OH})_2\text{D}$  analogs contributes to their diverse potency in suppressing PTH gene expression *ex vivo* [128]. However, all of these CKD-induced adverse molecular interactions are tissue- and gene-specific, not measurable *in vivo*, and consequently, do not provide appropriate therapeutic targets.

Fig. 79.2 presents a previously unrecognized synergy between  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  for VDR activation that could be exploited to safely counteract CKD-induced impairments in the formation and transcriptional function of the  $1,25(\text{OH})_2\text{D}$  (analog)/VDR complex, thus improving the health benefits of VDR activation in CKD without escalating  $1,25(\text{OH})_2\text{D}$  (analog) dosage. Studies in the CYP27B1-null mouse [129], which lacks the enzyme that converts  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$ , and *in vitro* studies using  $25(\text{OH})\text{D}$  analogs chemically modified to prevent hydroxylation at carbon 1 [130,131], have conclusively demonstrated that  $25(\text{OH})\text{D}$  activates the VDR directly and also synergizes with  $1,25(\text{OH})_2\text{D}$  activation of the VDR, as will be described by the efficacy of the  $25(\text{OH})\text{D}$ /paricalcitol combination to overcome parathyroid VDR resistance to suppress PTH and parathyroid cell growth in a rat model of hyper-phosphatemic kidney disease [117].

The concurrence of vitamin D and  $1,25(\text{OH})_2\text{D}$  deficiency with VDR reductions in CKD patients makes this synergy a valuable tool to improve clinical outcomes without escalating  $1,25(\text{OH})_2\text{D}$  (analogs) doses. Furthermore, since 25-hydroxylases are ubiquitously distributed and are not as tightly regulated as the constitutive renal CYP27B1 and CYP24A1 enzymes, local  $25(\text{OH})\text{D}$  production following cholecalciferol supplementation could safely counteract the reductions in intracellular levels of  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  or of the  $1,25(\text{OH})_2\text{D}$ /VDR complex induced by CKD with minimal impact on serum  $25(\text{OH})\text{D}$  levels or systemic calcium and phosphorus homeostasis.

The next section updates our understanding of the most critical vitamin D actions to attenuate CKD progression and improve skeletal and cardiovascular outcomes with the goal of identifying accurate and sensitive early biomarkers of the efficacy of vitamin D interventions to increase survival with minimal risk of toxicity.

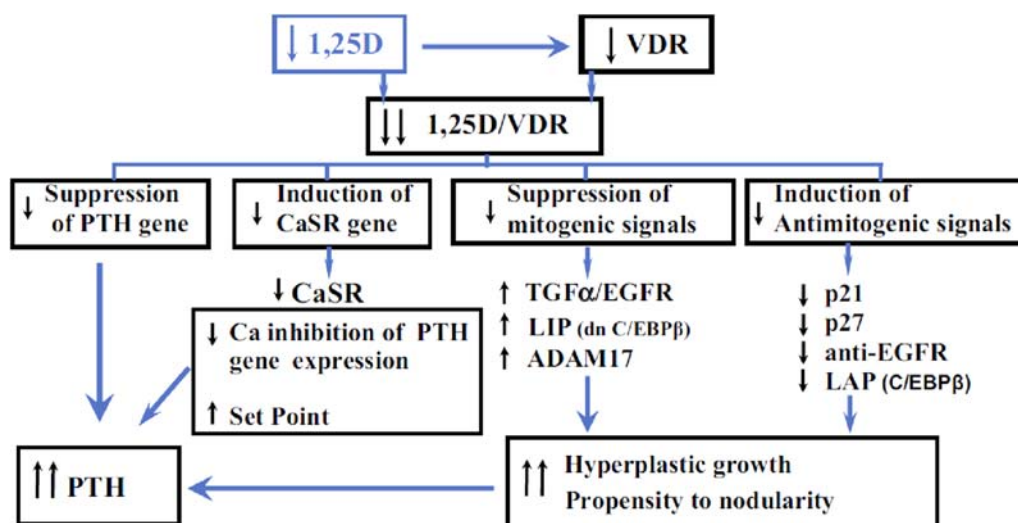
## 7. Vitamin D control of secondary hyperparathyroidism

Nearly all patients with end-stage renal disease develop SHPT, a condition characterized by parathyroid hyperplasia and increased synthesis and secretion of PTH, which severely affects skeletal and vascular health, markedly increasing morbidity and mortality rates. Three decades of experience with the clinical challenge of counteracting the onset of parathyroid resistance to either  $1,25(\text{OH})_2\text{D}$  (analogs) replacement therapy or vitamin D supplementation has resulted in

the parathyroid gland being one of the best characterized targets of the vitamin D endocrine–autocrine/paracrine system (summarized in Figs. 79.2 and 79.6) (see Chapter 17).

Hypocalcemia, hyperphosphatemia due to phosphate retention, vitamin D, and  $1,25(\text{OH})_2\text{D}$  deficiency are the main causes of SHPT [132]. The transcriptional repression of the PTH gene by the  $1,25(\text{OH})_2\text{D}$ /VDR complex involves its binding to a “classical” negative VDRE on the PTH gene promoter [133]. The elevations in serum PTH levels in vitamin D–deficient individuals with normal kidney function [50] as well as the reductions in serum PTH with vitamin D supplementation at early stages of kidney disease [103] or in renal transplant recipients [45] all support a role for parathyroid  $1\alpha$ -hydroxylase and autocrine/paracrine VDR activation in PTH suppression (see Fig. 79.2).

Vitamin D and/or  $1,25(\text{OH})_2\text{D}$  deficiency also impair the response of the parathyroid gland to calcium due to reductions in parathyroid content of the calcium-sensing receptor (CaSR), as demonstrated in vitamin D–deficient rats [134] and CKD patients [135]. The CaSR gene is directly induced by  $1,25(\text{OH})_2\text{D}$  through VDR/RXR binding to VDREs in this gene promoter [136]. Accordingly,  $1,25(\text{OH})_2\text{D}$  treatment increases the CaSR content reducing the set point for PTH suppression by calcium [137]. Significantly,  $1,25(\text{OH})_2\text{D}$  upregulation of the CaSR plays a critical role in attenuating hyperphosphatemia-induced PTH secretion. In 2019, the CaSR was identified as the “phosphate sensor” [138]. Increases in serum phosphate concentrations, within the pathophysiological range of CKD, suppress CaSR activity through noncompetitive antagonism that stabilizes the CaSR molecule in an inactive



**FIGURE 79.6** Role of  $1,25(\text{OH})_2\text{D}$  deficiency in the pathogenesis of secondary hyperparathyroidism in chronic kidney disease. *CaSR*, calcium-sensing receptor; *CKD*, chronic kidney disease; *EGFR*, epidermal growth factor receptor; *PTH*, parathyroid hormone; *TGF*, transforming growth factor; *VDR*, vitamin D receptor.

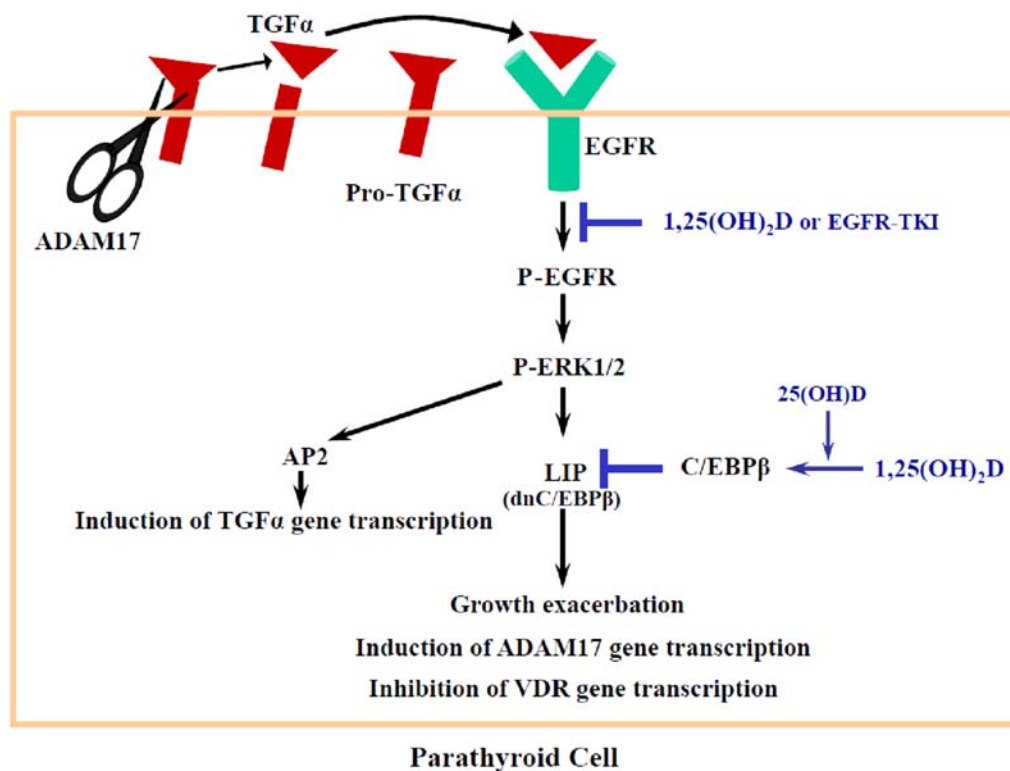
conformation, leading to chronically stimulated PTH release. It is therefore possible that variations in the high serum phosphate in advanced CKD would differentially displace the CaSR toward an inactive conformation. This may partially explain the high variability in the response to calcimimetic drugs, which are CaSR agonists, to suppress PTH in hyperphosphatemic states.

1,25(OH)<sub>2</sub>D induction of FGF23 synthesis in bone cells of the osteoblast/osteocyte lineage provides an additional indirect mechanism for vitamin D suppression of PTH secretion and parathyroid hyperplasia in CKD, provided there is sufficient parathyroid or circulating klotho [139], another gene that is induced by the 1,25(OH)<sub>2</sub>D/VDR complex [13] and reduced in the parathyroid glands of CKD patients. FGF23 induction of parathyroid CYP27B1 mRNA levels [98] may also contribute to autocrine/paracrine PTH suppression.

Prolonged persistence of hypocalcemia or vitamin D deficiency induces parathyroid cell proliferation to meet the higher requirements of serum PTH to normalize serum calcium. Hyperphosphatemia also

stimulates parathyroid hyperplasia [140]. In turn, the hyperplastic growth results in not only higher serum PTH levels but also marked reductions in the parathyroid VDR, CaSR, FGF receptors, and cell membrane klotho, all of which impair PTH suppression by active vitamin D, oral calcium, or the early increases in FGF23 [141].

The increased TGF $\alpha$  content noted in parathyroid adenomas and diffusely hyperplastic and nodular glands from CKD patients [142] led to identification of the essential role of TGF $\alpha$  activation of its receptor, the EGFR, in the severity of parathyroid hyperplasia and VDR reductions [143]. Briefly, as summarized in Fig. 79.7, the release of mature TGF $\alpha$  from its transmembrane precursor by ADAM17, an enzyme essential for parathyroid gland development [144], initiates a powerful autocrine loop for excessive TGF $\alpha$ /EGFR-growth signals because TGF $\alpha$  induces its own gene expression [145] and that of the ADAM17 gene [144], in part through increases in AP2 and LIP, respectively. In turn, the increases in the oncogene LIP (also



**FIGURE 79.7** Pathogenesis of parathyroid hyperplasia and 1,25(OH)<sub>2</sub>D resistance in CKD and its reversal by combined vitamin D and 1,25(OH)<sub>2</sub>D administration. Increases in parathyroid ADAM17, ADAM17-mediated release of TGF $\alpha$ , TGF $\alpha$  activation of the EGFR, and TGF $\alpha$ /EGFR induction of the synthesis of AP2, an inducer of TGF $\alpha$  gene expression, and of the potent mitogen LIP (the dominant negative isoform of C/EBP $\beta$ , dn C/EBP $\beta$ ) causes a feed forward loop for exacerbated growth and propensity to nodular hyperplasia as LIP directly enhances ADAM17 gene transcription. Because LIP also exerts a dominant negative inhibition of VDR gene transcription, the reductions of parathyroid VDR content causing resistance to calcitriol therapy are directly proportional to the severity of the hyperplastic growth. 1,25(OH)<sub>2</sub>D suppresses activated-EGFR signaling as effectively as EGFR-tyrosine kinase inhibitors (EGFR-TKI). 25(OH)D synergizes 1,25(OH)<sub>2</sub>D induction of C/EBP $\beta$  counteracting a powerful vicious cycle for exacerbated growth and VDR reductions. CKD, chronic kidney disease; EGFR, epidermal growth factor receptor; TGF, transforming growth factor; VDR, vitamin D receptor.

called dominant negative C/EBP $\beta$ ) are responsible for the suppression of VDR gene expression [143]. The concurrent overexpression of proliferating signals, such as TGF $\alpha$ , ADAM17, and LIP, may contribute to the transformation of parathyroid growth from diffuse to nodular [146]. The essential role of EGFR activation in CKD-driven parathyroid hyperplasia, TGF $\alpha$  self-induction, and VDR reductions has been conclusively demonstrated by the parathyroid phenotype of nephrectomized mice harboring a parathyroid-specific EGFR inactivation [144].

1,25(OH) $_2$ D/VDR suppression of parathyroid cell growth involves transactivation of the growth suppressors p21 and p27 [147], and of C/EBP $\beta$ , a growth inhibitor and a trans-activator of the VDR gene [148]. The complex also promotes “non-classical” control of TGF $\alpha$ /EGFR-driven hyperplasia by regulating subcellular trafficking with sequestration of activated EGFR in early endosomes [149]. As a dominant negative C/EBP $\beta$ , LIP antagonizes C/EBP $\beta$  function so that the cellular C/EBP $\beta$ /LIP ratio determines the prevalence of growth arrest or excessive growth. Although the 1,25(OH) $_2$ D/VDR complex directly induces the expression of the C/EBP $\beta$  gene [148], the main regulation of cellular C/EBP $\beta$ /LIP ratios is at the level of translation of either molecule from a single C/EBP $\beta$  mRNA. TGF $\alpha$ /EGFR growth signals induce the translation of LIP over that of C/EBP $\beta$ , which results in LIP-driven induction of ADAM17 gene expression [117] and reduction of the VDR gene [143]. The efficacy of rapamycin, an mTOR inhibitor, in suppressing parathyroid hyperplasia [121] supports a role for the 1,25(OH) $_2$ D/VDR control of mTOR [120] in counteracting the abnormalities in the translational control of parathyroid C/EBP $\beta$ /LIP ratio in advanced CKD. The activation of mTOR induced by the onset of renal failure or hypercalcemia is central for the development of SHPT and parathyroid cell proliferation in a rat model of adenine-induced renal failure [121].

1,25(OH) $_2$ D/VDR suppression of ADAM17 gene expression, a key target “upstream” of EGFR activation, contributes to the synergy between 1,25(OH) $_2$ D and the EGFR-specific tyrosine kinase inhibitor erlotinib in controlling parathyroid hyperplasia in 5/6 nephrectomized rats fed high dietary phosphorus [117]. Importantly, in this rat CKD model, the erlotinib/1,25(OH) $_2$ D synergy, which cannot be used therapeutically in renal patients as erlotinib is prescribed for EGFR-driven carcinomas, can be safely mimicked by the intraperitoneal administration of a combination of 25(OH)D and the 1,25(OH) $_2$ D analog paricalcitol (Fig. 79.7). Briefly, a dose of 25(OH)D that corrects vitamin D deficiency but fails to control SHPT combined with a dose of paricalcitol also insufficient to suppress either serum PTH or parathyroid cell growth reduced serum PTH by 50%

and suppressed parathyroid ADAM17 as effectively as phosphorus restriction [117] despite similar serum calcium and phosphorus levels. Mechanistically, improved parathyroid 1,25(OH) $_2$ D synthesis and the synergy between 25(OH)D and 1,25(OH) $_2$ D for VDR activation can partly explain the higher efficacy of this combination to suppress PTH and inhibit parathyroid ADAM17. These findings may partially explain why the exclusive correction of vitamin D deficiency could be insufficient to reduce serum PTH even at early CKD stages [50]. Also, vitamin D supplementation may benefit advanced CKD patients unresponsive to therapy, whose mild elevations in serum calcium and phosphorus impede escalating 1,25(OH) $_2$ D (analogs) dosage.

The oral formulations for cholecalciferol and active vitamin D therapy currently available may compromise the safety of the intraperitoneal route used in rat CKD studies. Specifically, the same 25(OH)D/active vitamin D synergy for intestinal VDR activation will increase calcium and phosphorus absorption aggravating hypercalcemia or hyperphosphatemia. Intestinal 25(OH)D/paricalcitol (1,25(OH) $_2$ D) synergy and CKD-induced impairments in 1,25(OH) $_2$ D (analogs) catabolism despite increased 24-hydroxylase mRNA levels [88,89] will markedly increase the risk of hypercalcemia and hyperphosphatemia. A safer option would be to escalate the dosage of vitamin D supplements to increase parathyroid 25(OH)D synthesis while reducing or maintaining existing 1,25(OH) $_2$ D (analogs) doses to avoid vitamin D toxicity.

For decades, the safe control of serum PTH by 1,25(OH) $_2$ D (analogs) to avoid hypercalcemia, hyperphosphatemia, and PTH over-suppression has been the treatment of choice for renal osteodystrophy. The goal is to prevent hyperphosphatemia and high PTH-induced increases in arterial calcium deposition promoting hypertension, cardiac overload, LVH, and high risk of cardiovascular mortality [7]. The COSMOS study, a large prospective study with a 3 year follow-up of the clinical handling of CKD-5D patients throughout Europe, has provided a range within the U-shaped curve for serum PTH that correlates with the lowest relative risk of mortality [150]. The clinical relevance of this PTH range was further corroborated by the survival benefits of correcting serum PTH to values within this range. Therefore, 1,25(OH) $_2$ D/analog suppression of PTH was also considered a mediator of the improvements in cardiovascular outcomes. However, 1,25(OH) $_2$ D (analog) induction of serum FGF23, a direct stimulus of pathological LVH through its activation of FGFR4-calcineurin pathway in cardiomyocytes [14], has suggested that prevention of the elevations of FGF23 would be more effective than PTH suppression to control LVH and cardiovascular risks in advanced CKD. Indeed, a very recent study by Dorr and



collaborators has compared the efficacy of etelcalcetide, a calcimimetic drug indicated for SHPT, with that of the VDR agonist alpha-calcidol, which is activated to 1,25(OH)<sub>2</sub>D in the liver, at controlling changes in LVH after a 12 month treatment [151]. In the primary intention to treat analysis, the changes in left ventricular mass index favored etelcalcetide over alpha-calcidol. Furthermore, the magnitude of the changes in LVMI was independently associated with FGF23 reductions [151], suggesting that reductions in FGF23 rather than PTH suppression could better estimate the cardiovascular risk in ESRD patients [152]. However, it is important to consider the multiple determinants of high FGF23 in CKD unrelated to 1,25(OH)<sub>2</sub>D (analog) therapy and, more significantly, that 1,25(OH)<sub>2</sub>D (analog) could further improve calcimimetic amelioration of serum FGF23 by (1) Reducing FGFR4 expression in cardiomyocytes to impair FGF23/FGFR4 induction of LVH [153]; (2) Maintaining circulating s-klotho to force FGF23 to switch from a klotho-independent binding to FGFR4-driven signaling to a s-klotho-dependent formation of a protective s-klotho/FGFR1/FGF23 complex [17], (3) Inducing the expression of the CaSR to ameliorate hyperphosphatemia-induced displacement of the CaSR toward an inactive conformation, thus improving calcimimetic suppression of PTH [136,138].

The next section highlights 1,25(OH)<sub>2</sub>D/VDR actions for skeletal protection in CKD unrelated to an effective control of SHPT.

## 8. Vitamin D control of skeletal health in CKD unrelated to the attenuation of secondary hyperparathyroidism

In CKD, the combination of vitamin D and/or 1,25(OH)<sub>2</sub>D insufficiency/deficiency, the onset of resistance to vitamin D therapy due to low VDR in bone cells, high PTH, and reduced renal klotho contribute to a wide range of bone disorders that predispose to vascular calcifications and increased risk for cardiovascular mortality. Several landmark studies have demonstrated the essential role of vitamin D for normal skeletal development and mineralization since the initial discovery that vitamin D deficiency was the cause of rickets, more than a century ago. However, the subtle abnormalities in bone mineral density in the VDR-null mouse fed an adequate supply of calcium and phosphorus (the so-called rescue diet) suggested that the VDR was dispensable for the ossification process [154]. Comprehensive studies in the VDR-null, 1 $\alpha$ -hydroxylase-null, and PTH-null mice, as well as multiple double knockout combinations, have revealed the essential role of 1,25(OH)<sub>2</sub>D for the induction of osteoblastogenesis,

skeletal anabolism, and the appropriate coupling of osteoblastic and osteoclastic activity [155,156].

The 1,25(OH)<sub>2</sub>D/VDR complex regulates the expression of genes that control bone formation, mineralization and remodeling (osteopontin, osteocalcin, and the Wnt receptor LRP5), and osteoclastogenesis and bone resorption (RANK ligand (RANKL) and osteoprotegerin (OPG)) through classical genomic actions (reviewed in Ref. [133]). In health, the physiological or supra-physiological concentrations of serum 1,25(OH)<sub>2</sub>D determine the prevalence of bone formation over resorption. In addition, the stage of differentiation of cells of the osteoblastic lineage determines 1,25(OH)<sub>2</sub>D actions. 1,25(OH)<sub>2</sub>D anabolic and anti-catabolic properties prevail in more mature cells, as conclusively demonstrated by overexpression of the VDR in mature osteoblasts in vivo [157]. Therefore, the coexistence of all of these distinct cell maturation stages in bone may partially explain the existing controversies regarding the prevalence of 1,25(OH)<sub>2</sub>D anabolic or catabolic actions in bone.

In mouse and rat CKD models, 1,25(OH)<sub>2</sub>D and its analog paricalcitol similarly maintained bone anabolism but differed in their osteoclastogenic potency [158,159] through a distinct regulation of several critical genes for bone remodeling. High 1,25(OH)<sub>2</sub>D potently induces RANKL and suppresses the expression of its decoy receptor OPG amplifying resorptive signals. Defective induction of osteopontin by the 1,25(OH)<sub>2</sub>D/VDR complex in CKD could adversely impact remodeling and osteoclast recruitment to resorb ectopic bone (reviewed in Ref. [133]). Similarly, an impaired induction of osteocalcin could negatively affect bone strength and energy metabolism through osteocalcin-mediated insulin release [160].

The accumulation of the Wnt inhibitors sclerostin and Dkk1 during CKD progression [161] generated great interest in CKD-induced defects in Wnt signaling in osteocytes and osteoblasts. This process is critical for skeletal development and mineralization, and consequently, is a potential contributor to the strong association between bone loss and increased vascular calcification [162]. Impaired 1,25(OH)<sub>2</sub>D induction of the LRP5 gene, involved in Wnt pathway activation, could contribute to bone loss in CKD. Although LRP5 induction by the VDR appears to occur regardless of 1,25(OH)<sub>2</sub>D VDR binding [124], CKD also reduces VDR content and its biological half-life.

A comprehensive examination of the mechanisms for the onset of abnormalities in Wnt pathway activation from early CKD stages has questioned the higher value for measurements of serum sclerostin levels over serum PTH in estimating skeletal damage [161] and has also revealed additional mechanisms for skeletal protection by a normal vitamin D status. Specifically, studies in a

mouse model of polycystic kidney disease, which reproduces a slow progression of human CKD, have demonstrated that the impaired Wnt activation in bone occurs before elevations in serum PTH [163]. Furthermore, in both mouse and rat CKD models, bone sclerostin decreases below the level of sham-operated controls as serum PTH and FGF23 increase. Clearly, this early increase in bone sclerostin is independent of the severity of SHPT. Instead, the progressive loss of bone mass in these experimental models was paralleled by elevations in bone levels of several Wnt inhibitors other than sclerostin, including Dkk1 [163–165]. Accordingly, bone biopsies in CKD patients corroborated a strong Wnt inhibition despite a lower number of osteocytes positive for sclerostin [163], thus questioning the accuracy of serum sclerostin to reflect not only the degree of Wnt inhibition in bone but also bone sclerostin levels. These studies have also demonstrated an unprecedented direct role for high FGF23 on Wnt inhibition in osteoblasts through the induction of Dkk1 [164].

The prevention of the early increases in bone sclerostin and loss of bone mass in this mouse CKD model with a TGF $\beta$  antibody [165] suggested that CKD induces early increases in TGF $\beta$  to promote Wnt inactivation. Therefore, a normal vitamin D status could help attenuate CKD-induced onset of adverse TGF $\beta$ /Smad signaling in bone, as VDR signaling antagonizes TGF $\beta$ /Smad-dependent transcriptional activation of several profibrotic genes through the recruitment of VDR to loci on these genes that prevent/attenuate Smad3 binding [166]. An important additional benefit of vitamin D–dependent regulation of the Wnt pathway is that it is tissue-specific. For instance, in the kidney and the vasculature where Wnt activation worsens the progression of renal damage and vascular calcification, vitamin D inhibits Wnt signals [167,168] through VDR binding to  $\beta$ -catenin in the cytosol preventing its translocation to the nucleus [118,169].

Another controversial issue is whether 1,25(OH) $_2$ D induction of FGF23 synthesis in bone could act as an autocrine–paracrine mineralization-regulating factor in osteocytes and osteoblasts since both FGF23 excess and deficiency lead to inhibition of bone formation and mineralization [170]. These opposing FGF23 effects are partly explained by either suppression (in the case of FGF23 excess) or induction (in the case of FGF23 deficiency) of tissue non-specific alkaline phosphatase (TNAP) in a klotho-independent manner (reviewed in Ref. [171]). While suppression of TNAP leads to accumulation of pyrophosphate, a mineralization inhibitor, the high release of inorganic phosphate by increased TNAP results in the induction of osteopontin, another mineralization inhibitor [171]. As mentioned, FGF23 also impairs bone formation through the induction of the Wnt inhibitor Dkk1 [164].

The role of reductions of bone cell klotho in CKD in the maintenance of skeletal integrity is also a highly controversial issue. Indeed, contrary to the reductions in bone density and cortical bone thickness in the klotho hypomorphic mouse, specific ablation of the klotho gene in osteocytes resulted in increased osteoblastic activity and bone formation rates [172]. Since klotho is the most powerful inducer of FGF23 in bone, it is possible that klotho deletion prevents/attenuates the deleterious impact of high FGF23 on bone mineralization. Intriguingly, despite the markedly reduced osteocyte klotho levels in an experimental mouse CKD model, there were no improvements in bone density [172], suggesting that CKD also impairs the normal function of the FGF23/klotho axis to protect the bone.

The next section addresses CKD-induced alterations of the FGF23-klotho/1,25(OH) $_2$ D/phosphate axis.

## 9. Vitamin D maintenance of renal FGF23/klotho axis: relevance for renal and cardiovascular protection

1,25(OH) $_2$ D/VDR-mediated regulation of FGF23 and klotho expression to maintain a normal bone–kidney–parathyroid FGF23/klotho axis is crucial for survival. In individuals with normal renal function, 1,25(OH) $_2$ D/VDR transactivation of the FGF23 gene in osteocytes and osteoblasts facilitates the pro-survival actions of vitamin D via two mechanisms. First, FGF23 promotes the renal elimination of phosphorus to prevent hyperphosphatemia and its pro-aging consequences. Second, it suppresses renal 1,25(OH) $_2$ D synthesis and induces 25(OH)D and 1,25(OH) $_2$ D catabolism in the kidney and non-renal cells to avoid vitamin D toxicity. In fact, the main features of the FGF23-null mouse are hyperphosphatemia, high circulating 1,25(OH) $_2$ D, ectopic calcifications, premature aging, arteriosclerosis, and osteoporosis [173], a severe phenotype that can be rescued by dietary phosphorus restriction [68,174,175]. It should be noted that hyperphosphatemia enhances serum FGF23 by increasing the half-life of the FGF23 protein through binding to FGFR1, resulting in upregulation of the *galnt3* gene, which is responsible for the O-glycosylation of full-length FGF23 that impedes its intracellular inactivating cleavage [24].

The low serum FGF23 in VDR- and CYP27B1-null mice [176] suggests that an impaired induction of FGF23 during vitamin D deficiency contributed to their accelerated pro-aging features and mortality rates. However, because double knockouts of FGF23 and either the VDR [177] or CYP27B1 [178] could rescue the severe pro-aging features of the FGF23-null mice by preventing hyperphosphatemia, 1,25(OH) $_2$ D induction of FGF23 is

nowadays considered an adverse effect of 1,25(OH)<sub>2</sub>D replacement therapy in CKD.

In the past 6 years, it has become clear that 1,25(OH)<sub>2</sub>D replacement is not the main reason for the abnormally high FGF23 levels in advanced CKD. Several factors, unrelated to alterations in bone and mineral metabolism, contribute to increased serum FGF23 in CKD (reviewed in Ref. [16]). These include reductions in dentin matrix protein I (DMP1) expression, the enzyme responsible for the cleavage of FGF23 for its inactivation [179]; hypoxia, a direct inducer of FGF23 gene transcription and stability [180]; increased release of glyceraldehyde 3 phosphate by the acutely damaged kidney, which induces bone FGF23 production [181]; and systemic inflammation [182], with LPS, IL-1 $\beta$ , and TNF being the main inducers of FGF23 gene transcription [183].

Significantly, inflammatory signals affect FGF23 production not only in bone but also in the thymus and spleen [184], and, in the case of LPS, FGF23 induction also occurs despite hypo-phosphatemic conditions. Instead, high 1,25(OH)<sub>2</sub>D fails to trans-activate the FGF23 gene under hypo-phosphatemic conditions, as demonstrated in a transgenic mouse with an ablation in the gene for the phosphorus transporter NPT2a [185]. Furthermore, neither hyperphosphatemia nor PTH, the latter inducing FGF23 gene transcription through the same enhancer utilized by inflammatory cytokines [168], is able to increase serum FGF23 if serum calcium is below 4 mg/dL [23].

The plethora of FGF23 inducers in CKD suggests that 1,25(OH)<sub>2</sub>D therapy is not the sole contributor to increased LVH risk associated directly with the high serum FGF23 in these patients. To the contrary, it suggests that 1,25(OH)<sub>2</sub>D anti-inflammatory actions could attenuate the elevations of circulating FGF23 induced by inflammatory cytokines. Also, 1,25(OH)<sub>2</sub>D-mediated reduction in FGFR4 [153] should attenuate the kloθο-independent FGF23-driven PLC $\gamma$ /calcineurin/NFAT pathway involved in promoting LVH [14,17].

1,25(OH)<sub>2</sub>D/VDR induction of the longevity gene  $\alpha$ -kloθο is essential for FGF23 phosphaturic [186] and PTH-suppressing actions [187], which require kloθο to form a stable ternary complex with FGF23 and FGFR1c to promote FGF23 signaling. Indeed, the early and progressive reductions in renal kloθο in the course of CKD progression contribute to the onset of renal resistance to FGF23 phosphaturic actions. Studies in CKD patients' stage 3–4 have demonstrated that progressive reductions of renal kloθο associate not only with a reduced fractional excretion of phosphate in response to FGF23 but also with a four-fold higher propensity for abdominal aortic calcification [188].

In opposition to our initial understanding that FGF23 resistance in CKD was limited to the few tissues

harboring kloθο, including the kidney, the parathyroid gland, and the choroid plexus [189], crystal structure analysis has now revealed soluble kloθο as a component of the FGF23/FGFR1c complex [17]. This recent finding provides a mechanism by which hyperphosphatemia is avoided and normal 1,25(OH)<sub>2</sub>D levels are maintained despite high FGF23 levels in a mouse model harboring a conditional kloθο deletion in renal proximal tubular cells, where phosphate reabsorption and 1,25(OH)<sub>2</sub>D synthesis take place [69].

Soluble kloθο (sKloθο) is released to the circulation upon cleavage of the transmembrane 140 kDa full-length kloθο molecule by the proteases ADAM17 and ADAM10 [190]. sKloθο may attenuate the pathological impact of high FGF23 in the heart of CKD patients through its binding to FGFR4 to simultaneously increase FGF23 binding affinity and block the kloθο-independent PLC $\gamma$ /calcineurin/NFAT pathway responsible for cell contractility, altered intracellular calcium, and hypertrophic cell growth. Instead, kloθο-dependent RAS/MAPK signaling is protective against adverse cardiac events [17]. sKloθο attenuation of FGF23 pathological actions could also extend to the amelioration of renal phosphate excretion through the binding of residual FGFR1 molecules in proximal tubular cells to increase FGF23 binding affinity and phosphaturic signaling [17].

Undoubtedly, the progressive reductions in renal content of the longevity gene kloθο also increase mortality rates in the course of CKD due to accelerated skeletal, immune, renal, and cardiovascular aging through FGF23-independent mechanisms [191]. Because a kidney-specific kloθο ablation mimicked the accelerated skeletal, immune, renal, and cardiovascular aging phenotype of the kloθο-null mice [192] and a VDRE was identified in the human kloθο promoter [13], a defect in vitamin D induction of renal kloθο was assumed to mediate, at least in part, the epidemiological association between vitamin D deficiency and higher risk of all-cause mortality in the general population [52], a risk that is markedly exacerbated in CKD patients [10]. Indeed, increased mortality rates were identified in hemodialysis patients carrying a kloθο polymorphism that impaired function. Moreover, survival rates were improved by 1,25(OH)<sub>2</sub>D (analog) administration [193], but it is unclear if the increased survival was the result of elevations in the defective renal kloθο or of direct pro-survival actions of active vitamin D therapy unrelated to renal kloθο induction.

sKloθο exerts a wide range of cellular functions beyond the tight regulation of mineral metabolism, which contribute to renal and cardiovascular protection [194] and increased survival [26,29]. These actions include inhibition of Wnt signaling, apoptosis, inflammatory signaling, fibrosis, as well as induction of

autophagy, preservation of stem cells, and maintenance of endothelial cell function to avoid the development of hypertension. In mice with normal kidney function, klotho-induced autophagy by disruption of the formation of the Beclin1/Bcl2 complex [195] is one of the mechanisms critical for klotho prevention of premature aging and life span improvements, which are unrelated to the attenuation of hyperphosphatemia. Similar klotho-mediated increases in autophagic flow partly explain why systemic administration of recombinant klotho or intravenous injection of urinary vesicles rich in klotho [196] rescues the renal and cardiovascular damage associated to acute or chronic renal injury in mice [197–199]. Importantly, despite a complete recovery of renal function after AKI, there is a later progression to CKD. In mice, klotho/s-klotho induction of autophagic flow attenuates the progression of acute kidney injury (AKI) to CKD after either bilateral ischemia reperfusion injury or unilateral ischemia with unilateral nephrectomy [200]. Indeed, while klotho haploinsufficient mice progressed to CKD much faster, klotho overexpressing mice were protected, an effect also mimicked by administration of recombinant  $\alpha$ -klotho that was associated with increases in renal cell autophagic flow. However, the contribution of the antioxidant, anti-apoptotic actions of sKlotho cannot be ruled out.

In diabetic kidney disease, decreases in serum klotho associate with the severity of albuminuria and renal insufficiency [201]. Conversely, klotho overexpression in spontaneously diabetic mice halts kidney disease progression [202,203]. Klotho-mediated inhibition of the Wnt/ $\beta$ -catenin pathway is the main mechanism by which kidney disease progression is avoided in diabetes [204]. Significantly, the administration of a small peptide of 30 amino acids, comprising the Gln186 to His 215 of the klotho molecule to a mouse model of streptozotocin-induced diabetes type 1 or to genetic db/db type 2 diabetic mice, was sufficient to attenuate glomerular hypertrophy, podocyte injury, glomerulosclerosis, and interstitial fibrosis without significant changes in serum calcium or phosphate levels [205]. The actions of this mini-klotho molecule involve inhibition of  $\beta$ -catenin activation without affecting Wnt expression. Specifically, the small klotho peptide binds Wnt ligands decreasing their ability to interact with lipoprotein receptor-related protein 5/6 (LRP5/6), thereby impeding LRP6 phosphorylation and  $\beta$ -catenin activation.

Since excessive Wnt signaling contributes to osteogenic differentiation of vascular smooth muscle cells promoting arterial calcification [206], the benefits of klotho-derived peptides may also extend to vascular protection. Thus, in diabetes, klotho-derived peptides provide a potentially effective approach to alleviate the

failure of current therapies aimed at improving glycemic control or inhibiting the renin–angiotensin–aldosterone system or sodium glucose co-transporter 2 to slow renal progression to end-stage renal disease.

Soluble klotho also acts at the apical site of the renal tubule as a glucuronidase enzyme to induce phosphaturia, urinary K excretion, and calcium reabsorption (reviewed in Ref. [207]). Different from the FGF23/FGFR1/klotho complex that decreases phosphate cotransporters at the cell surface [175], sKlotho cleaves residues that promote the endocytosis of the sodium–phosphate cotransporter NPT2a, thereby impeding phosphorus entrance into tubular cells provided there is sufficient megalin [208].

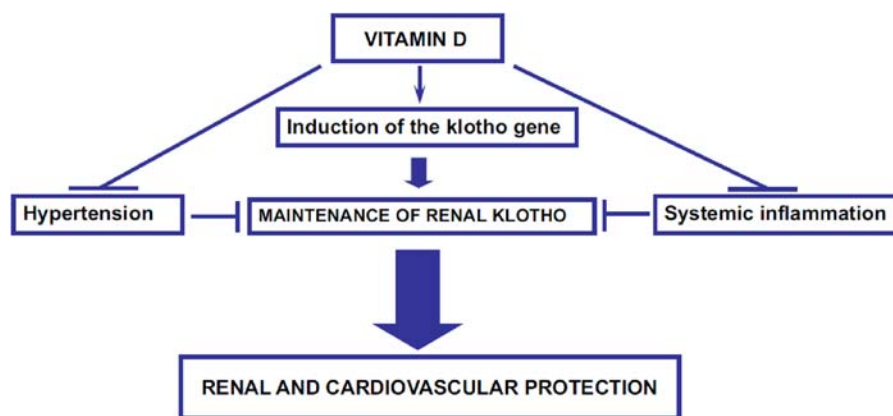
Circulating klotho also induces posttranslational modifications on the calcium channel TRPV5 to fix the protein to the cell surface enhancing renal calcium uptake [209] and leading to a positive bone calcium balance [210]. The recently solved crystal structure of the FGF23/FGFR/klotho complex has excluded the klotho sialidase activity, thought to mediate sKlotho modulation of TRPV5 and the K channel ROMK1, and suggested that sKlotho may act as a lectin that binds specific sugar residues in these channels to target them to the apical membrane [29]. Defective maintenance of renal TRPV5 activity due to impaired klotho transcytosis to the urinary side by a failing kidney could contribute to the direct correlation between serum klotho higher than normal, negative calcium balance, and increased incidence of fractures.

Overall, preventing the reduction of renal and/or circulating klotho is essential to reduce the severity of CKD progression and the risk for cardiovascular mortality. Indeed, serum s-klotho decreases with age, incidence of hypertension [211], and systemic inflammation [212] all recognized determinants of renal damage and cardiovascular disease. Vitamin D also maintains renal klotho content through the control of hypertension and systemic inflammation (Fig. 79.8).

The main contribution of the kidney to serum s-klotho levels was strongly supported by an 80% reduction in circulating s-klotho upon specific ablation of renal klotho [192]. This finding was critical to consider that serum s-klotho levels could be an accurate biomarker of renal klotho content, CKD progression, and cardiovascular mortality risk in CKD patients. However, the kidney is also the main organ for the clearance of circulating klotho into the urine, through a process of transcytosis through tubular cells [213]. Therefore, increases in circulating s-klotho could reflect an impaired transcytosis to the urine by the damaged kidney, which may mask both actual renal klotho reductions and potential improvements in renal klotho content induced by vitamin D interventions. Also, current assays fail to discriminate full-length klotho from



**FIGURE 79.8** Vitamin D maintenance of renal klotho contributes to renal and cardiovascular protection. Vitamin D simultaneous induction of the klotho gene and inhibition of hypertension and systemic inflammation maintains renal klotho content, thereby ensuring klotho renal and cardiovascular protection.



shorter circulating isoform of unknown biological activity. Therefore, serum and urinary s-klotho are not accurate biomarkers of renal and cardiovascular risk at present. Improvements in the available assays and in sample preservation are mandatory first steps before establishing adequate ranges for serum and urinary s-klotho that are associated with the lowest mortality risk at different CKD stages [26].

As vitamin D restriction ameliorates the severe phenotype of the klotho-null mouse [67] and klotho suppresses CYP27B1 expression [68], avoiding excessive vitamin D interventions is essential for the proper functioning of this strong counter-regulatory loop between vitamin D and klotho to ensure survival, a challenge aggravated in CKD by an impaired vitamin D catabolism despite increased cyp24A1 expression [88,89].

### 10. Vitamin D control of hypertension and systemic inflammation conferring renal and cardiovascular protection independently of renal or systemic klotho levels

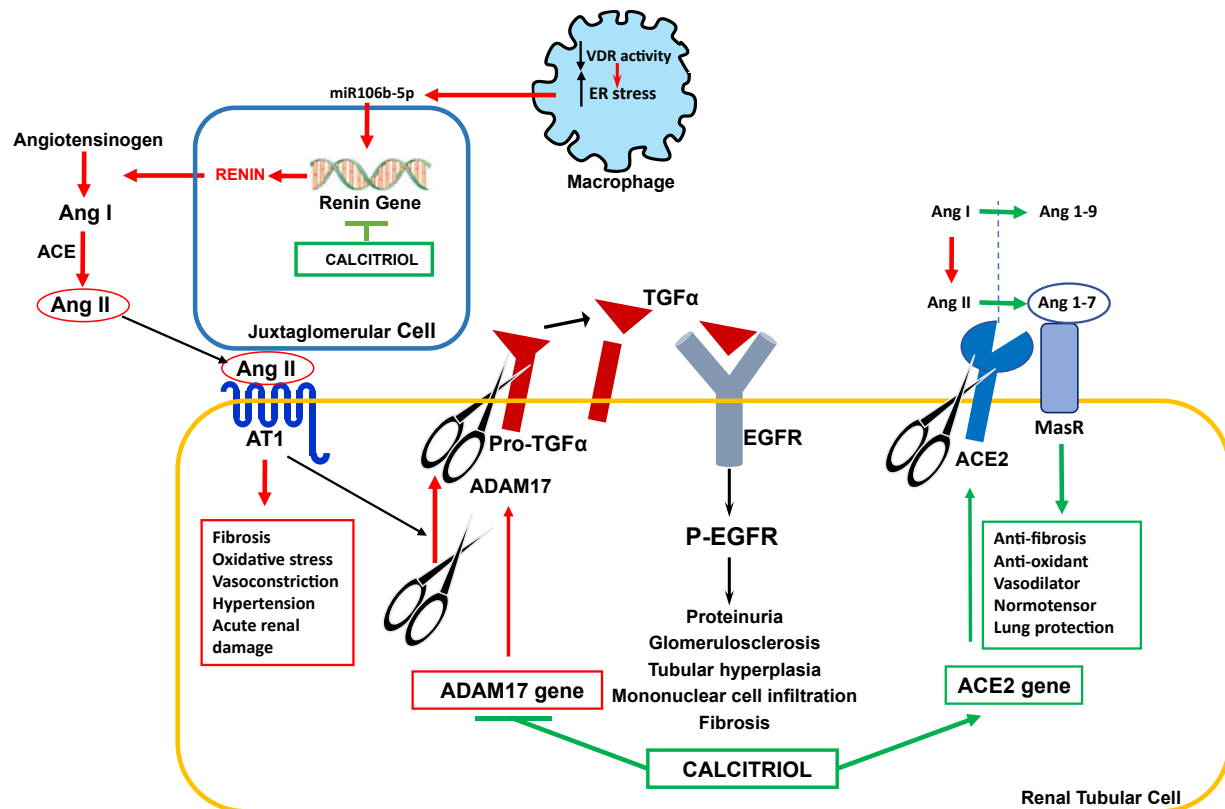
Vitamin D deficiency has been associated with the development of hypertension. 1,25(OH)<sub>2</sub>D/VDR inhibition of the renin gene [214] mediates in part the causal association between increases in circulating 25(OH)D levels and reductions in blood pressure and hypertension demonstrated by Mendelian randomization studies [215]. However, the results from randomized controlled trials in individuals with normal renal function are controversial, partly due to the inclusion of subjects with normal vitamin D levels or receiving anti-hypertensive medications as well as variable vitamin D interventions [216].

Vitamin D downregulation of renin production persists in renal disease. Simultaneous administration of the angiotensin receptor 1 (AT1R) inhibitor losartan and the 1,25(OH)<sub>2</sub>D analog paricalcitol in rodent models

of diabetic nephropathy effectively attenuated losartan-induced increases in renin, resulting in lower serum angiotensin II [217]. Downregulation of RAAS by 1,25(OH)<sub>2</sub>D (analog) therapy has also shown effective in reducing proteinuria, systemic inflammation, and cardio-renal syndrome progression [7]. However, the 1,25(OH)<sub>2</sub>D (analog)/VDR complex ameliorates angiotensin II-driven renal damage independently of its downregulation of renin-driven RAAS activation through the suppression of ADAM17 expression and the induction of the angiotensin-converting enzyme II (ACE2) gene expression, as summarized in Fig. 79.9.

Lautrette and collaborators have conclusively demonstrated that the renal damage after a prolonged exposure to high angiotensin II is caused by angiotensin II-induced translocation of ADAM17 from the cytosol to the cell membrane of renal proximal tubular cells to release TGF $\alpha$ . This process results in EGFR activation, causing tubular hyperplasia, fibrosing glomerulosclerosis, proteinuria, inflammatory infiltration to the renal parenchyma, and stabilization of cytosolic ADAM17 [218]. In fact, TAPI II, an ADAM17 inhibitor, as well as specific ablation of the EGFR in proximal tubular cells, markedly attenuated the development of severe renal lesions despite persistent hypertension. This mechanism is of high clinical relevance because, in a normal kidney, ADAM17 is expressed at low levels exclusively in the distal convoluted tubules; however, human CKD of any etiology markedly increases renal ADAM17/TGF $\alpha$  expression and signaling throughout the kidney [219].

Once the increased renal ADAM17 initiates the release of TNF $\alpha$  into the circulation, TNF $\alpha$  induces ADAM17 gene transcription [220], resulting in a positive feedback loop to further exacerbate renal ADAM17 expression, the release of TGF $\alpha$  driving renal damage, and TNF $\alpha$ -driven systemic inflammation and inflammatory renal damage. These processes are not responsive to anti-RAAS therapy but still suppressible by 1,25(OH)<sub>2</sub>D (analogs)-mediated inhibition of ADAM17 [117] and



**FIGURE 79.9** Antihypertensive properties of the 1,25(OH)<sub>2</sub>D–VDR complex. In juxtaglomerular cells, 1,25(OH)<sub>2</sub>D (calcitriol) suppression of renin gene expression ameliorates systemic hypertension by reducing circulating angiotensin II levels. In macrophages, decreased VDR activation leads to ER stress–dependent secretion of miRNA 106b-5p, which induces juxtaglomerular cell renin production. In renal tubular cells, calcitriol opposes angiotensin II–driven renal damage by inhibiting ADAM17-mediated increases in TGF/EGFR signals and inducing ACE2 expression to promote angiotensin 1–7/MAS receptor-mediated anti-hypertensive, anti-fibrotic, and anti-inflammatory signals.

TNFα gene expression (Fig. 79.9). Thus, combined suppression of renin and ADAM17 gene expression by 1,25(OH)<sub>2</sub>D may contribute to the synergy between paricalcitol and the angiotensin II–converting enzyme (ACE) inhibitor enalapril in reducing the inflammatory macrophage infiltration to the renal parenchyma in rat CKD [221].

In the vasculature, 1,25(OH)<sub>2</sub>D-mediated suppression of the ADAM17/TNFα loop should decrease TNFα-induction of vascular neutral sphingomyelinase-2 expression and activity, responsible for the release of pro-calcifying exosomes to propagate calcium deposition in the medial arterial layer [222].

1,25(OH)<sub>2</sub>D induction of ACE2 gene expression effectively counteracts angiotensin II–driven hypertensive, inflammatory, and pro-fibrotic signals, as depicted in Fig. 79.9. ACE2 catalyzes the conversion of both angiotensin I and angiotensin II into angiotensin 1–9 and angiotensin 1–7, respectively, thereby reducing circulating angiotensin II levels and ameliorating its deleterious renal and cardiovascular effects that follow RAAS hyper-activation [223]. In addition, angiotensin 1–7 binding to its receptor MAS activates anti-fibrotic,

anti-oxidant, and vasodilatory signals favoring multi-organ protection and normotension [224,225].

Since ADAM17 cleaves ACE2, the increased ADAM17 expression and activity of kidney disease patients may partly account for the strong correlation between increases in circulating ACE2 and classical cardiovascular risk factors, such as older age, diabetes, and male gender, all associated with a higher risk for cardiovascular events, in CKD patients with no history of cardiovascular damage [226]. Thus, the cardiovascular protection by 1,25(OH)<sub>2</sub>D and its analogs may result from the combination of ADAM17 suppression and ACE2 induction. In fact, in non-obese diabetic mice, the 1,25(OH)<sub>2</sub>D analog paricalcitol, alone or in combination with aliskiren, a direct renin inhibitor, effectively counteracted the rise in circulating soluble ACE2 levels associated with diabetes. Reduced ADAM17 expression, oxidative stress, and proteinuria [227] were also observed, emphasizing the renoprotective effects of ACE2 induction.

1,25(OH)<sub>2</sub>D also induces the expression of microRNA-145 (miR-145) [228], the prevalent microRNA in vascular smooth muscle cells (VSMC) and a

master regulator of vascular smooth muscle cell fate. Increases in miR-145 provide a previously unrecognized mediator of vitamin D protection against vascular injury with aging and kidney disease progression, as nephron reduction and hyperphosphatemia decrease aortic miR-145 content [229]. Importantly, miR145 overexpression increases, and its silencing reduces the expression of alpha-actin and markers of the integrity of the arterial elastin layer in the absence of pro-calcifying signals, supporting the relevance of vitamin D induction of miR-145 in maintaining proper arterial contractility and preventing osteogenic differentiation even under non-calcifying stimuli [115]. Reduced VSMC miR-145 promotes loss of the contractile phenotype with de-differentiation toward a proliferative, migratory phenotype causing a blunted hypertensive response, arterial thickening, restenosis, and atherosclerosis [230–233].

Hyperphosphatemia, a risk factor for VC and cardiovascular mortality in individuals with normal renal function [234], also reduces miR-145 content in normal aortas [235]. In turn, miR-145 downregulates the osterix gene [236], an inducer of bone mineralization [237], and directly targets the transcription factor Krüppel-like factor 4 (KLF4), which controls the expression of osteogenic Runx2 [238–240], thus supporting a role for miR145 reductions in vitamin D deficiency- or hyperphosphate-mia-driven vascular calcification. Importantly, combined therapy with 25(OH)D and paricalcitol in a mouse model of established uremia was sufficient to prevent reductions of aortic miR-145, thereby attenuating decreases in  $\alpha$ -actin and increases in Runx2 despite severe hyperphosphatemia [115].

In addition to its anti-hypertensive properties, the integrity of the vitamin D endocrine system is essential to ensure immune protection from bacterial and viral infections, minimize progression of autoimmune disorders, and avoid excessive inflammation [241]. The 1,25(OH)<sub>2</sub>D/VDR complex influences innate and acquired immunity through multiple mechanisms including decreasing the antigenicity of antigen-presenting cells, downregulating the production of pro-fibrotic and pro-inflammatory Th1 cytokines, enhancing the production of anti-inflammatory Th2 cytokines, shifting T cell polarization toward a Th2/regulatory phenotype, and stimulating T regulatory lymphocyte development [1,241] (see Chapter 96). 1,25(OH)<sub>2</sub>D inhibition of Skp2 expression may partly explain the maintenance of T regs [242] and the blockage of autophagic flow driven by the cell invasion by Middle East respiratory syndrome coronavirus (MERS-CoV) [243]. 1,25(OH)<sub>2</sub>D immune regulatory properties confer protection not only from inflammation-driven multiple organ damage but also from inflammation-driven reductions of renal klotho that accelerate CKD progression.

In CKD, increased ADAM17-mediated release of TNF $\alpha$  to the circulation is a main determinant of the degree of systemic inflammation and renal and cardiovascular damage, as demonstrated by a higher degree of cardiovascular mortality in individuals with normal renal function carrying a polymorphism of ADAM17 that results in a mild increase in TNF $\alpha$  release [244]. Furthermore, specific deletion of ADAM17 in myeloid cells is sufficient to markedly reduce mortality rates in a rat model of LPS-induced sepsis [245]. Accordingly, in hemodialysis patients, circulating mononuclear cells express higher levels of ADAM17 and higher serum levels of TNF $\alpha$ , which can be reduced by combined vitamin D and 1,25(OH)<sub>2</sub>D (analog) administration [246]. Significantly, similar to the impaired 25(OH)D uptake in renal proximal tubular cells with advancing CKD, peripheral blood mononuclear cells from hemodialysis patients also have a 40% reduced capacity for 25(OH)D uptake. Surprisingly, even when mononuclear cells do not express megalin, the defective uptake was corrected with 1,25(OH)<sub>2</sub>D administration [96].

Finally, as indicated earlier, the induction of ADAM17 activity at the cell membrane induced by elevations by circulating angiotensin II provides a causal link between hypertension and inflammation, two highly interrelated processes. Importantly, as depicted in Fig. 79.9, a novel causal link between inflammation-driven hypertension has been identified recently. In mice, while the absence of monocyte lineage prevents angiotensin II–driven hypertension [247], the over-activation of the renin–angiotensin system results in renal and vascular accumulation of pro-inflammatory macrophages, resulting in increased oxidative stress and its associated tissue damage. In the vasculature, hypertension-induced macrophage infiltration drives nitric oxide scavenging causing reductions in renal blood flow [248], which in turn induces renin secretion by juxtaglomerular cells [248]. However, until recently, there was no evidence for immune cell–induced hypertension or its modulation by vitamin D. Studies from the Bernal-Mizrachi laboratory have demonstrated that increases in ER stress in response to vitamin D deficiency are sufficient to cause renin–dependent hypertension through the secretion of micro-RNA 106b-5p that enables a direct communication between innate immune cells and juxtaglomerular cells [116]. Importantly, the miR106b-5p link between inflammatory immune cells and renin secretion by juxtaglomerular cells can be prevented by the 1,25(OH)<sub>2</sub>D/VDR complex.

In summary, the integrity of the vitamin D endocrine system protects from CKD progression through direct inhibition of renal klotho reductions, maintenance of the FGF23/sklotho axis, attenuation of systemic inflammation, and hypertension that ameliorate CKD-induced excess of circulating FGF23. Maintenance of renal klotho

ensures the reduction of the pro-aging actions of phosphate retention, as well as the longevity properties of klotho-mediated induction of renal auto-phagic flow and of klotho and klotho-derived peptide inhibition of Wnt/ $\beta$ -catenin signaling to attenuate the progression of diabetes to diabetic nephropathy, as well as novel actions of the FGF23/sklotho/FGFR1 ternary complex to counteract FGF23 pathological actions in the heart. However, circulating soluble klotho is not an accurate marker of renal klotho reductions.

Vitamin D-mediated suppression of hypertension has a dual impact on CKD progression by reducing angiotensin II-driven renal damage and attenuating the reductions in renal klotho. The former is achieved through the maintenance of an adequate balance between pro-hypertensive (renin and ADAM17) and anti-hypertensive signals (ACE2). Increases in circulating ACE2 levels may provide an accurate measurement of increased ADAM17 activity and the cardiovascular risk associated with reductions in ACE2 expression at the cell membrane in CKD, which may be corrected with 1,25(OH)<sub>2</sub>D (analog) therapy. Finally, 1,25(OH)<sub>2</sub>D-mediated reduction of macrophage miR106b-5p release into the circulation provides the first causal link between macrophage ER stress activation and the induction of renin-dependent hypertension.

Measurements of miR106b, circulating ACE2, angiotensin II, or angiotensin 1–7 levels rather than circulating 25(OH)D levels may help improve vitamin D supplementation strategies to overcome the tremendous variability among individuals in their capacity not only for local 1,25(OH)<sub>2</sub>D production, but also for the local bio-activation of vitamin D to 25(OH)D in tissues bearing 25-hydroxylases that limit vitamin D renal and cardiovascular protection.

### **11. Progress to improve current recommendations for the evaluation of vitamin D status and for the design of safe and effective vitamin D interventions**

This section presents an update of recent international efforts to present physicians with evidence-based clinical practice recommendations to comply with the 2017 KDIGO guidelines to correct vitamin D deficiency in CKD prior to the initiation of 1,25(OH)<sub>2</sub>D (analog) interventions to treat SHPT and ameliorate cardiovascular risks [111].

The National Kidney Foundation (NKF) gathered a panel of experts at a Controversy Conference on Vitamin D in CKD3-4 in 2018 to help resolve current controversies on the diagnosis of vitamin D deficiency/insufficiency and provide definitive indications for optimal therapeutic intervention for its correction to control

elevations in serum PTH [249]. Briefly, the consensus was to consider (1) Serum 25(OH)D concentrations above 20 ng/mL in these patients as “adequate” vitamin D levels, with no need for supplementation; (2) Serum 25(OH)D below 15 ng/mL as an indication of vitamin D deficiency that requires supplementation regardless of serum PTH, and (3) Serum 25(OH)D levels in the 15–20 ng/mL range as an indicator of vitamin D insufficiency, with no need for supplementation unless there is evidence of counter-regulatory hormone activity (increased PTH or FGF23).

Similar recommendations emerged from a CKD-MBD working group from the European Society of Pediatric Nephrology for CKD2-5 and dialysis patients (less than 18 years old), who are more prone to nutritional rickets, poor bone growth, and poor overall bone health [250]. These recommendations were applicable also for kidney transplant recipients, who have a higher propensity to bone abnormalities due to their prior CKD, the degree of SHPT after transplant, and the immunosuppressive therapy. In children, normal 25(OH)D has been shown sufficient to delay the onset of SHPT and to prevent nutritional rickets. Therefore, this group recommended supplementation to patients with vitamin D deficiency (25(OH)D < 20 ng/mL) or vitamin D insufficiency (25(OH)D > 20 ng/mL, < 30 ng/mL) with ergocalciferol or cholecalciferol to achieve normal 25(OH)D (> 30 ng/mL). Importantly, supplementation strategies should be adjusted by age and baseline vitamin D status.

Due to the high variability between 25(OH)D assays, the European working group also recommends the use of external quality control strategies to ensure the accuracy of 25(OH)D<sub>2</sub> or 25(OH)D<sub>3</sub> measurements, as well as the assessment of serum and urinary calcium to minimize the risks for vitamin D toxicity from hypercalcemia, hypercalciuria, and nephrocalcinosis. In case of children with proteinuria, the group also recommended to evaluate bioavailable serum 25(OH)D by correcting for serum DBP and albumin levels [250].

Ergocalciferol, cholecalciferol, 25(OH)D, and extended release 25(OH)D are the compounds currently available for vitamin D supplementation. Ergocalciferol and cholecalciferol are equally effective at increasing serum 25(OH)D levels when administered daily [251]. The shorter half-life of 25(OH)D<sub>2</sub> compared with that of 25(OH)D<sub>3</sub> requires an increased frequency of D<sub>2</sub> dosage to compensate for its decay below basal levels by 2 weeks after bolus ergocalciferol administration [252]. High bolus doses of either compound should be avoided, not only for their higher risk of toxicity, but also because the rates of conversion of vitamin D to 25(OH)D decrease for dosages above 4000 IU [41].

The extended-release form of 25(OH)D (caldiol, ERC) has been available in the United States since 2016, and 25(OH)D has been the main compound for



the correction of vitamin D deficiency in Spain. Both compounds are extremely useful for the rapid correction of serum 25(OH)D, particularly in individuals with hepatic dysfunction or undergoing treatment with anti-epileptic medications with impaired conversion of vitamin D to 25(OH)D in the liver [253,254]. The efficacy to rapidly augment serum 25(OH)D may better counteract the impaired substrate availability to renal or parathyroid  $1\alpha$ -hydroxylases [85,92]. However, important safety considerations include the higher potency of 25(OH)D to induce intestinal calcium absorption and 1,25(OH)<sub>2</sub>D catabolism, even in individuals with normal renal function.

The recent randomized clinical trial in CKD3–4 patients demonstrating that extended release calcifediol (ERC) administration safely suppressed PTH only after achieving serum 25(OH)D concentrations higher than 50 ng/mL suggested that current targets for vitamin D repletion are too low [255]. However, these high concentrations were shown to increase the risk of mortality in women with normal kidney function and cause symptomatic toxicity in children with hypercalcemia and suppressed PTH.

Undoubtedly, it is unclear whether a supplementation strategy efficacious to correct vitamin D deficiency and suppress PTH will improve bone mineral density and bone strength and reduce fragility fractures. Indeed, in men and women 70 years or older with normal renal function and a prior fall, cholecalciferol supplementation of 24,000 IU monthly improves the incidence of non-vertebral fractures and frequency of falls due to improved muscular function and physical activity [256]. However, cholecalciferol dosage of 60,000 IU, or of 24,000 IU in combination with 300 ug of 25(OH)D reverses the benefits of correcting vitamin D deficiency causing an increase in the number of falls and impaired suppression of PTH. Most likely, induction of 1,25(OH)<sub>2</sub>D catabolism by the excess of 25(OH)D mediates these undesired actions.

To better control for an adverse impact on bone from vitamin D supplementation, both international working groups suggested to improve current evaluation of bone turnover (bone-specific and total alkaline phosphatase, serum collagen 1C telopeptide, intact procollagen type 1-terminal propeptide) to assess the adequacy of vitamin D interventions without bone biopsies.

Changes in proteinuria with vitamin D supplementation could provide a surrogate marker of the degree of CKD progression. Thus, the NKF Controversy Conference also suggested the implementation of intermediate end point trials to identify populations of responders, effective agents, and optimal doses before moving to clinical outcomes studies. It is also recommended to personalize vitamin D interventions using 24,25(OH)2D3/25(OH)D ratios and 1,25(OH)D/25(OH)D ratios,

serum PTH and FGF23 as surrogate markers of the abnormalities in the regulation of renal 1-hydroxylase and 24-hydroxylase activity with CKD progression.

In contrast to vitamin D supplementation strategies to control SHPT in CKD, there are precise recommendations for 1,25(OH)<sub>2</sub>D (analog) replacement therapy according to serum PTH, calcium, and phosphate levels as well as CKD stages. Therapeutic agents include 1,25(OH)<sub>2</sub>D, two prohormones  $1\alpha$ (OH)D<sub>2</sub> and  $1\alpha$ (OH)D<sub>3</sub>, which are activated to 1,25(OH)<sub>2</sub>D by the 25-hydroxylation by hepatic hydroxylases, and three analogs. The analogs include paricalcitol (19nor, 1,25-dihydroxyD<sub>2</sub>), maxacalcitol (22-oxa-1,25(OH)<sub>2</sub>D), and fale1,25(OH)<sub>2</sub>D (hexafluorinated 1,25(OH)<sub>2</sub>D). Nevertheless, individualized treatment plans to control SHPT with 1,25(OH)<sub>2</sub>D (analog) should be established and closely monitored to ensure the best possible outcomes while avoiding potential risks such as alterations in calcium and phosphate levels as well as insufficient inhibition or over-suppression of PTH.

Importantly, despite the survival benefits initially reported for paricalcitol over 1,25(OH)<sub>2</sub>D in hemodialysis patients and in numerous studies in animal models of CKD, at present there are no conclusive studies supporting the use of 1,25(OH)<sub>2</sub>D or analogs to improve survival.

## 12. Summary points

- In the normal kidney renal tubular cell, the highly coordinated, cell-specific genomic regulation of CYP27B1 and CYP24A1 by PTH, FGF23, 1,25(OH)<sub>2</sub>D, calcium, and phosphate ensures the maintenance of serum 1,25(OH)<sub>2</sub>D and 25(OH)D levels within normal levels.
- In CKD, vitamin D–mediated suppression of systemic inflammation contributes to the maintenance of renal and circulating anti-aging klotho and ameliorates the adverse impact of cytokine-induced elevations in renal, spleen, and immune cell FGF23 production.
- In CKD, vitamin D–mediated suppression of FGFR4 expression and induction of the klotho gene may counteract FGF23/FGFR4-driven LVH and the resistance to FGF23 phosphaturic action by facilitating ternary FGF23/s-klotho/FGFR1 complex formation.
- Small klotho peptides effectively control  $\beta$ -catenin activation by mimicking s-klotho to attenuate the progression of diabetic nephropathy.
- Defective vitamin D signaling in monocyte–macrophages increases the susceptibility of CKD patients to bacterial and viral infections and hypertension.

- In CKD, the correction of vitamin D deficiency with cholecalciferol or ergocalciferol supplementation should be personalized according to surrogate markers of vitamin D activation and catabolism and directed to improve clinical outcomes regardless of circulating 25(OH)D levels.

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# Vitamin D and kidney stones

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## OBJECTIVES

- Understand vitamin D metabolism as it relates to urinary calcium excretion and calcium kidney stone formation.
- Explore the relationship between vitamin D supplementation and kidney stone formation.
- Understand current approaches to the prevention and treatment of bone mineral disease in calcium kidney stone formers.

## 1. Introduction

Vitamin D is integral to calcium and phosphorus homeostasis and to bone metabolism (see Chapters 16–18). Its deficiency is associated with low bone mass and secondary hyperparathyroidism (see Chapters 62 and 63). The formation of calcium kidney stones is associated with higher urinary calcium concentrations, which may be increased by higher circulating levels of 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ). The potential risk that  $1,25(\text{OH})_2\text{D}$  can increase urinary calcium excretion, and thereby lithogenesis, has made clinicians hesitant to treat vitamin D deficiency in patients with a history of calcium stones. Treatment with vitamin D is commonly listed as a risk factor for calcium kidney stone disease [1]. However, observational data supporting this putative relationship are not compelling. The relationship between vitamin D and calcium stones remains controversial. Currently, there is no clear contraindication to treating vitamin D deficiency in patients with calcium kidney stones. However, whether screening for

vitamin D deficiency, followed by supplementation, in such patients (or in the general population) is even worthwhile is also not established.

## 2. Calcium kidney stones

Kidney stone disease is a major clinical and economic health burden with an estimated prevalence of about 10% in the US population. Its prevalence has increased in the United States over the past 30 years with a cost burden of more than \$5 billion annually in the United States [2,3]. Kidney stones are recurrent: after passage of a first stone, the risk of recurrence is 40% at 5 years and 75% at 20 years.

The most common type of kidney stones are composed of calcium, and they comprise 85% of all stones. Calcium stones are made of calcium oxalate (50%), calcium phosphate (~5%–10%), and a mixture of both (45%) [4]. They are associated with other chronic conditions such as hypertension, cardiovascular disease, metabolic syndrome, and diabetes mellitus. Evidence suggests that dietary habits, such as diets high in sodium and animal protein with fewer fruits and vegetables, are common risk factors for kidney stone formation and link stones with the aforementioned chronic diseases. Calcium stones are also commonly associated with bone mineral loss and fractures [5,6].

Calcium stone formation is a complex physiological process. The key factors are high urine supersaturation, crystallization, growth, and aggregation. In calcium stone formers, an imbalance between excretion of calcium, oxalate, citrate, and water results in crystal precipitation and aggregation [1]. Higher urine calcium excretion is a significant risk factor for calcium stone

formation. Since most stones contain calcium, and because higher urine calcium excretion has been associated with the formation of stones, a traditional treatment approach was dietary calcium restriction. However, this approach has now been out of favor for nearly 30 years. Patients with greater dietary calcium intake have fewer stones [7,8]. Patients treated with higher dietary calcium intake (and with lower intake of animal protein, sodium, and oxalate), compared with patients challenged by a low-calcium diet, had fewer stones [9]. Such out-of-date calcium-restricted diets would be expected to be associated with loss of significantly more calcium in the urine than control patients and result in a reduction in bone mineral density and increased rate of fracture [10]. Data have also suggested that a higher dietary calcium intake may decrease the incidence of symptomatic kidney stones and bone loss. The effect of higher dietary calcium intake on stone prevention is attributed to the binding of ingested calcium to oxalate in the intestinal lumen, leading to reduced intestinal oxalate absorption leading to lower urinary saturation of calcium oxalate [11].

### 3. Vitamin D metabolism

Vitamin D, obtained from diet or produced by skin, first undergoes hydroxylation in the liver by 25-hydroxylase (*CYP2PR1*) to yield 25-hydroxyvitamin D3 (25(OH)D), which is then further hydroxylated, primarily in the kidney, by 25(OH)D-1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase or *CYP27B1*) to produce bioactive form 1,25(OH)<sub>2</sub>D (see Chapter 4). The renal production of 1,25(OH)<sub>2</sub>D by 1 $\alpha$ -OHase is tightly regulated by parathyroid hormone (PTH), calcium, and phosphorus levels and by 1,25(OH)<sub>2</sub>D itself (see Chapter 8). The receptor for 1,25(OH)<sub>2</sub>D, vitamin D receptor (VDR), is located in the cell nucleus and found in most tissues, such as intestine, bones, kidneys, muscles, and organs of immune system (see Chapters 10–13).

The most important role of 1,25(OH)<sub>2</sub>D is the promotion of intestinal absorption of calcium. Decreased levels of 1,25(OH)<sub>2</sub>D diminish intestinal calcium absorption resulting in increased serum levels of PTH causing secondary hyperparathyroidism. Secondary hyperparathyroidism maintains normal serum calcium levels by increasing renal calcium resorption and thereby reducing 24 h urinary calcium excretion. 1,25(OH)<sub>2</sub>D also directly inhibits synthesis of PTH, regulates renal and intestinal phosphate absorption, and regulates osteoblastic function and bone resorption by stimulating fibroblast growth factor 23 (FGF23) [12] (see Chapter 19).

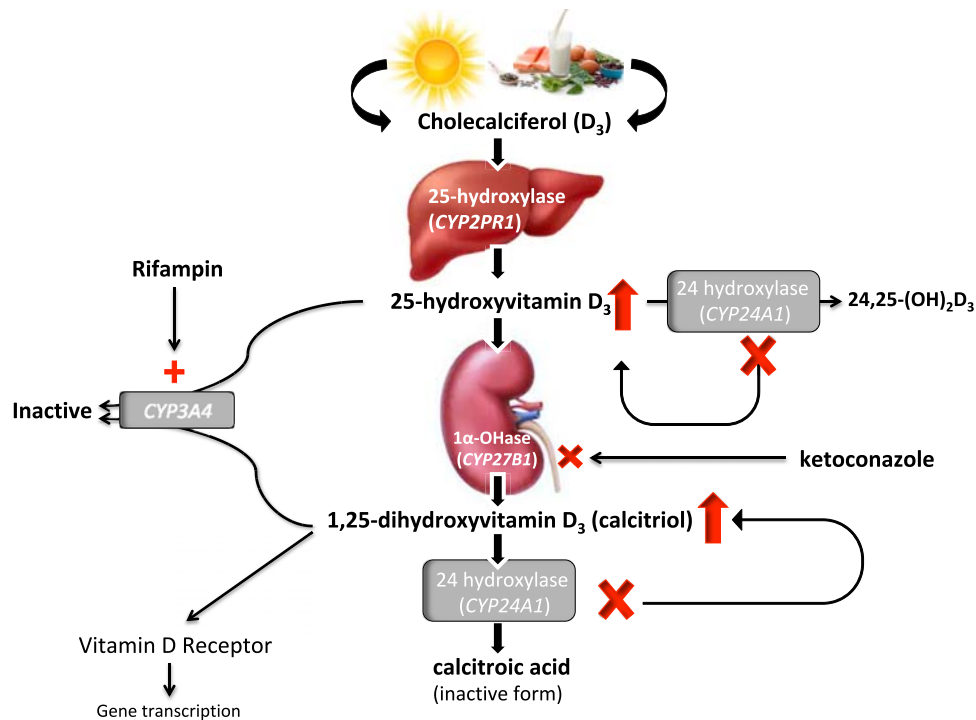
The enzyme 24-hydroxylase (24-OHase or *CYP24A1*) deactivates 1,25(OH)<sub>2</sub>D, forming 1,24,25-trihydroxy vitamin D, leading eventually to the production of calcitric acid [13]. In addition, *CYP24A1* converts 25(OH)D

to 24,25-dihydroxyvitamin D (24,25(OH)<sub>2</sub>D), which prevents its conversion to the active 1,25(OH)<sub>2</sub>D (see Chapter 5). Both calcium and 1,25(OH)<sub>2</sub>D inhibit the activity of 1 $\alpha$ -OHase. Therefore, the potential toxic effect of vitamin D supplementation to increase urinary calcium excretion may be mitigated: the effects of an increase in serum calcium and an increase in active vitamin D inhibit increases in 1 $\alpha$ -OHase activity [14]. As shown in Fig. 80.1, tight regulatory mechanisms are in place to control intestinal calcium absorption and limit increased urinary calcium excretion with vitamin D supplementation [15]. 1 $\alpha$ -OHase is upregulated by PTH, hypocalcemia, and hypophosphatemia and downregulated by 1,25(OH)<sub>2</sub>D. The deactivating enzyme, 24-OHase, is upregulated to cause degradation of 1,25(OH)<sub>2</sub>D and prevent hypercalcemia. Genetic variations in *CYP24A1* may explain why some calcium stone formers have elevated 1,25(OH)<sub>2</sub>D levels when supplemented with vitamin D [16].

### 4. Idiopathic hypercalciuria

Idiopathic hypercalciuria (IH) is a traditional term used for the frequent occurrence of higher urine calcium excretion in stone formers, the cause of which remains poorly elucidated. Even the term “hypercalciuria” is poorly defined, since no threshold for pathologic calcium excretion is widely recognized [17]. Patients with IH have an elevated urinary calcium excretion, are normocalcemic, and often have relatively elevated 1,25(OH)<sub>2</sub>D levels with normal PTH [18]. Calcium stone formers have higher urinary calcium excretion compared with nonstone formers regardless of dietary calcium intake. The distinction between “absorptive” and “renal” hypercalciuria is no longer considered appropriate, as features of both phenotypes are present in most such patients [19]. Similarly, low-calcium diets are not considered appropriate for the patients once called “absorptive” hypercalciuria. Higher urine calcium excretion has also been associated with low bone mineral density and fractures [5].

Observations suggest that the pathogenesis is due to a primary increase in intestinal calcium absorption and a primary overproduction of 1,25(OH)<sub>2</sub>D. Consistent with that hypothesis, studies have confirmed that on average, serum 1,25(OH)<sub>2</sub>D levels are higher in IH, and most interestingly, the changes in calcium metabolism can be induced by the administration of small doses of 1,25(OH)<sub>2</sub>D to healthy volunteers [20,21]. A prospective study following males over 12 years revealed higher plasma 1,25(OH)<sub>2</sub>D, even in ranges considered normal, and was independently associated with a higher risk of symptomatic kidney stones [22]. In fact, the mean value for serum 25(OH)D was in the normal range. The authors found that the association of serum 1,25(OH)<sub>2</sub>D with renal stone risk was



**FIGURE 80.1** Vitamin D metabolic pathways and activating and inhibiting factors associated with kidney stone formation.

independent of calcium intake and plasma levels of PTH, phosphorus, and 25(OH)D, all modulators of 1 $\alpha$ -OHase. In other words, the basis for these marginally higher values was uncertain. In addition, higher FGF23 levels were also associated with risk, although of borderline statistical significance, and of uncertain pathophysiology. Not all studies have been able to demonstrate an association between elevated serum 25(OH)D, in the range of 20–100 ng/mL, and kidney stones [23].

Two genes, soluble adenylate cyclase 10 (*ADCY10*) and vitamin D receptor (*VDR*), have been implicated in IH. Unlike *VDR*, the function of *ADCY10*, a bicarbonate-sensitive enzyme, and its link to urine calcium excretion, is not understood [24–26]. Likewise, the genetic hypercalciuric stone-forming rat (GHS) has been bred for higher urinary calcium excretion [27]. While the genes responsible for the elevated urinary calcium excretion remain uncertain, the model does recapitulate the human IH phenotype. Among the characteristics of this stone-forming animal model, elevated *VDR* expression is demonstrated in the intestinal mucosa, renal tubules, and bone cells [27]. These findings support the hypothesis that *VDR* may play a role in the human form of higher urine calcium as well. The GHS rat also recapitulates the human bone phenotype of lower bone mineral density and lower bone strength [28].

Inactivating mutations in *CYP24A1* lead to an inability to deactivate calcitriol. These mutations were first demonstrated in infantile hypercalcemia, an

inherited autosomal recessive disorder characterized by hypercalcemia and nephrocalcinosis (see Chapter 69). Because of the failure to inactivate vitamin D, PTH is suppressed and 1,25(OH)<sub>2</sub>D is elevated. Vitamin D supplementation was first implicated in the pathogenesis of this disorder during the epidemic of infantile hypercalcemia and increased doses of vitamin D in infant formula in the United Kingdom [14]. Less severe cases, presenting in adults, with stones, with or without hypercalcemia, are seen without significant vitamin D supplementation [29,30]. Typically, patients have high serum 1,25(OH)<sub>2</sub>D and low levels of 24,25(OH)<sub>2</sub>D. Nephrocalcinosis may lead to chronic kidney disease. The prevalence of *CYP24A1* mutations may account for a larger proportion of calcium stones among adults as well. In fact, this group of patients may account for some significant, but undefined proportion of patients, who are susceptible to kidney stones, particularly as a consequence of vitamin D supplementation. This finding is suggested by genome-wide association studies that do show that the *CYP24A1*-associated locus correlates with serum calcium concentration and the number of nephrolithiasis episodes [31]. These patients have been treated with ketoconazole and fluconazole, which inhibit 1 $\alpha$ -OHase, reducing serum levels of 1,25(OH)<sub>2</sub>D [32]. In addition, rifampin, the antituberculosis antibiotic, has been used successfully to inactivate vitamin D via induction of another catabolic enzyme *CYP3A4* [23] (see Chapter 67).



## 5. Vitamin D supplementation and kidney stone risk

Observational studies of individuals without kidney stones taking vitamin D supplements have not convincingly demonstrated that such supplementation is associated with increased urinary calcium excretion or increased rates of kidney stones.

Supplemental administration of vitamin D to attain circulating levels of 25(OH)D greater than 32 ng/mL in a group of 138 participants did not significantly increase urinary calcium excretion [33]. One prevalent limitation of such studies is that those in whom vitamin D is given without calcium supplementation are few in number, so that the role of vitamin D per se is unclear. Among stone formers as well, vitamin D repletion for a limited duration does not appear to adversely affect overall 24-h urine calcium excretion in individuals with vitamin D deficiency or insufficiency. A subset of individuals may have an increase in urinary calcium excretion, but this may be attributed to dietary habits such as high sodium and protein intake [33].

Two recent studies have demonstrated no statistically significant risk with vitamin D supplementation and kidney stone formation. In one study, 29 participants with a history of nephrolithiasis, urinary calcium excretion between 150 and 400 mg/d, and a serum 25(OH)D level less than 30 ng/mL were given oral ergocalciferol at a dose of 50,000 IU/week for 8 weeks. While serum 25(OH)D levels increased after repletion, mean 24-h urinary calcium excretion did not. Although 11 participants did have an increase in their urinary calcium excretion, urinary sodium excretion was also increased suggesting a dietary effect [33]. In the largest observational study to date on vitamin D and kidney stones, a prospective analysis of nearly 200,000 male and female participants from 3 well-characterized cohorts, Health Professionals Follow-up Study and Nurses' Health Study I and II, found no statistically significant association between vitamin D intake and risk of stones in men or women after multivariate adjustment (for different variables, including use of calcium supplements) [34]. Few in the cohort had vitamin D intake greater than 2000U daily, and the study concluded that vitamin D in "typical amounts" is not associated with increased risk of kidney stone formation. Among healthy postmenopausal women, calcium with vitamin D supplementation resulted in a small but significant improvement in hip bone density but did not significantly reduce hip fracture risk with an increased reported risk of kidney stones [35]. This is the only known study to report an increased risk of kidney stones after the administration of vitamin D. Again, this increased risk could be explained by the concomitant use of vitamin D with

elemental calcium, which is known to increase urinary calcium excretion. The US Preventative Services Task Force has found insufficient evidence to recommend screening in men and postmenopausal women and recommends against daily supplementation of 400IU or less of vitamin D and 1000 mg or less of calcium for the primary prevention of fractures in postmenopausal women. To date, the overall evidence regarding the effect of vitamin D in causing stones has been largely unconvincing.

## 6. Conclusions

Because vitamin D deficiency has been associated with cardiovascular disease, fractures, colorectal cancer, diabetes, depression, and death, the hope had been for vitamin D supplementation to reverse these adverse outcomes. However, randomized controlled trials have shown that vitamin D supplementation has low to no benefit on mortality, bone mineral density, and incidence of falls and fractures, diabetes, cancer, and cardiovascular disease [36]. In fact, recommendations now are relatively neutral, if not negative regarding vitamin D screening [36]. Compounding the difficulty of screening and interpretation of results are the variability between assay methods and the prevalent seasonal variation in 25(OH)D levels [37].

There is also a lack consensus about the definition of 25(OH)D deficiency and repletion regimens. As a result, the US Preventative Services Task Force recommends against the routine screening of vitamin D in asymptomatic adults who "are not known to have signs or symptoms of vitamin D deficiency or conditions for which vitamin D treatment is recommended" [38]. With regard to bone health, symptomatic patients with nontraumatic fractures, kidney dysfunction or malabsorptive disorders, including kidney stone patients, require intervention. A history of kidney stones, itself, is independently associated with higher risk of wrist fracture and a risk for osteoporosis [39]. Workup for metabolic abnormalities in kidney stone patients has also revealed inadequate vitamin D levels, making this population prone to being prescribed vitamin D supplementation [40]. However, as the evidence for vitamin D supplementation remains unsatisfactory, how can we treat bone mineral disease in at-risk stone formers? A prescription for dietary alteration is recommended. The Borghi diet, which recommends increased calcium intake and restricted sodium and animal protein intake, has been proven in a randomized clinical trial to prevent calcium stones [9]. Nondietary calcium supplementation, which frequently accompanies vitamin D supplementation, is positively associated with kidney stone

formation, while the inverse has been shown to be true for dietary calcium and kidney stone risk [8]. Calcium when administered as calcium citrate instead of calcium carbonate, or the timing of supplements with meals, may decrease the risk of lithogenicity when supplementation is required [41].

Thiazides are well established for the prevention of calcium stones and are recommended by both the American Urological Association and European Association of Urology. A metaanalysis revealed a 47% reduction in relative risk of calcium stone recurrence with thiazide use [42]. Thiazides increase calcium absorption in the distal tubule and indirectly in the proximal tubule reducing urinary calcium excretion. As a result, retained calcium increases bone mineral density and leads to a reported 10% reduction in fractures [43].

Hypocitraturia is a common finding in the workup of metabolic abnormalities in stone formers. Chronic acid loads in the form of high-protein diets, which reduce urinary pH and citrate excretion, are not only associated with kidney stone formation, but also with decreased bone mass [44]. The administration of oral potassium citrate increases urinary citrate and reduces calcium excretion, thereby reducing the risk of kidney stone formation, and has also been shown to increase bone mass in postmenopausal women [45].

Bisphosphonates are a well-established therapy for osteoporosis. In patients with low bone density, bisphosphonate use is associated with decreased 24-h urinary calcium excretion [46], and a lower incidence of kidney stones [47].

## 7. Summary points

- Vitamin D is integral to calcium balance and maintenance of bone health. Widespread screening for vitamin D deficiency and supplementation does not appear to have contributed to kidney stone prevalence in the general population. Vitamin D supplementation may increase stone risk in patients with underlying CYP24A1 mutations.
- There is no clear contraindication or benefit to treating vitamin D deficiency and bone mineral disease in patients with calcium kidney stones. Alterations in diet, thiazides, and citrate administration are instead well-established therapies for the prevention of calcium kidney stones.

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# Hypercalcemia due to vitamin D toxicity

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## OBJECTIVES

- Categorize the types of vitamin D toxicity.
- Explain the different mechanisms of vitamin D toxicity.
- Describe the clinical manifestations of vitamin D toxicity.
- Review diagnosis and treatment measures of hypercalcemia due to vitamin D toxicity.

## 1. Introduction

Vitamin D toxicity is an uncommon cause of hypercalcemia. In the differential diagnosis of hypercalcemia, it is often buried amid a long list of other more and less common causes (Table 81.1). The most common causes of hypercalcemia, among the many etiologies, are primary hyperparathyroidism (PHPT) and hypercalcemia of malignancy, accounting for more than 90% of cases. Other causes of hypercalcemia, such as vitamin D toxicity, granulomatous disorders, cosmetic injections, thyrotoxicosis, adrenal insufficiency, milk alkali syndrome, medications, familial hypocalciuric hypercalcemia, and immobilization, comprise less than 10% of all causes of hypercalcemia. It is important to consider these uncommon etiologies when PHPT and malignancy have been ruled out [1]. In these settings, vitamin D toxicity becomes an important consideration in the differential diagnosis of hypercalcemia. This chapter reviews the various forms of vitamin D toxicity,

mechanisms of hypercalcemia due to vitamin D toxicity, clinical manifestations, diagnosis, and management.

## 2. Forms of exogenous vitamin D toxicity

Vitamin D toxicity can be life-threatening and associated with substantial morbidity, if not identified quickly. Hypervitaminosis D with hypercalcemia may be secondary to excessive intake of parent vitamin D, its metabolites 25-hydroxyvitamin D (25(OH)D), 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D<sub>3</sub>), or vitamin D analogs; to increased production of 25(OH)D or 1,25(OH)<sub>2</sub>D<sub>3</sub> from exogenous substrate; and even to topical applications of potent vitamin D analogs. Ergocalciferol is metabolized to 1,25(OH)<sub>2</sub>D<sub>3</sub>, however, since exogenous calcitriol is 1,25(OH)<sub>2</sub>D<sub>3</sub> and many supplements are vitamin D<sub>3</sub>, for simplicity purposes we use the nomenclature “1,25(OH)<sub>2</sub>D<sub>3</sub>” to refer to all forms of calcitriol.

### 2.1 Vitamin D and 25(OH)D toxicity

The most common etiology of vitamin D toxicity is inadvertent or improper oral use of pharmaceutical preparations. Excessive ingestion of vitamin D, usually much greater than 10,000 IU per day, can cause vitamin D intoxication that is recognized by markedly elevated levels of 25(OH)D (typically >200 ng/mL) in association with levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> that are normal or only slightly elevated. Hyperphosphatemia can be a clue to the etiology of the vitamin D-mediated hypercalcemia since parathyroid hormone (PTH)-related and PTH-related peptide (PTHrP)-related disorders usually are



**TABLE 81.1** Differential diagnosis of hypercalcemia.

Primary hyperparathyroidism
Sporadic (adenoma, hyperplasia, or carcinoma)
Familial
Isolated
Cystic
Multiple endocrine neoplasia type I or II
Malignancy
Parathyroid hormone-related protein
Excess production of 1,25-dihydroxyvitamin D
Other factors (cytokines, growth factors)
Disorders of vitamin D
Exogenous vitamin D toxicity – parent D compound, 25(OH)D, 1,25(OH)D, vitamin D analogs
Endogenous production of 25-hydroxyvitamin D (may be seen in Williams syndrome)
Endogenous production of 1,25-dihydroxyvitamin D
Granulomatous diseases
a. Sarcoidosis
b. Tuberculosis
c. Histoplasmosis
d. Coccidioidomycosis
e. Leprosy
f. Others
Foreign body inflammatory reactions
Lymphoma
Non-parathyroid endocrine disorders
Thyrotoxicosis
Pheochromocytoma
Acute adrenal insufficiency
Vasoactive intestinal polypeptide hormone-producing tumor (VIPoma)
Medications
Thiazide diuretics
Lithium
Estrogens/antiestrogens, testosterone in breast cancer
Milk-alkali syndrome
Vitamin A toxicity
Familial hypocalciuric hypercalcemia
Immobilization
Parenteral nutrition
Aluminum excess
Acute and chronic renal disease

associated with normal or reduced serum phosphate levels [2–4]. However, it is important to recognize that hyperphosphatemia is not always seen in vitamin D toxicity, and there have been case reports of hypervitaminosis D–induced hypercalcemia associated with hypophosphatemia [5,6]. The usual setting of vitamin D toxicity is in its use as a therapy for hypocalcemic disorders such as hypoparathyroidism, pseudohypoparathyroidism, osteomalacia, or renal failure. Ingestion of excessive quantities of 25(OH)D, 1 $\alpha$ -hydroxyvitamin D, 1,25(OH)<sub>2</sub>D<sub>3</sub>, dihydrotachysterol, or exuberant use of the topical calcipotriene (Dovonex) for psoriasis can also cause vitamin D intoxication [7]. Health-conscious adults have been reported to ingest large doses of megavitamins from over-the-counter supplements, in amounts that may exceed 2 million IU of vitamin D daily

[8,9]. Cancer patients, in particular, have been observed to consume excess nutritional supplements such as calcium, vitamin D, and shark cartilage [10]. Hypercalcemia due to high doses of vitamin D use has also been reported in patients with multiple sclerosis [11–13]. Moreover, individuals have mistakenly ingested ergocalciferol 50,000 IU daily instead of more typical biweekly or monthly administration [6,14,15].

An over-the-counter supplement called Soladek has been implicated in vitamin D toxicity in a published case report and our own personal experience. This supplement, readily available in the Dominican Republic and in urban areas such as Manhattan, contains over 500,000 IU of vitamin D<sub>3</sub> and 120,000 IU of vitamin A per 5 mL vial. The package label for Soladek lists a number of indications for its use, including “hypo and avitaminosis, rickets, growth, dentition, lactation, fractures, infections, convalescence, protection and regeneration of certain epithelium (bronchial, glandular, ocular, cutaneous), corticotherapy, aging, pregnancy.” In the case report by Leu et al. [16], a 60-year-old woman with a medical history significant only for osteoarthritis presented with symptoms of hypercalcemia and a serum calcium of 15.2 mg/dL in the setting of recent Soladek use. Her 25(OH)D was >150 ng/mL with undetectable PTH and PTHrP levels. CT of the chest, abdomen, and pelvis, skeletal survey, and bone marrow biopsy were all negative for malignancy. However, colonoscopy revealed an anal squamous cell carcinoma that was resected. The patient’s hypercalcemia resolved after discontinuation of Soladek in addition to treatment with intravenous fluids, intermittent furosemide, and a single dose of pamidronate. In our own case series of nine patients, the majority of patients with hypercalcemia in the setting of Soladek use had a second condition that could have contributed to the development of hypercalcemia. No patient had previously experienced hypercalcemia before ingesting Soladek, however, indicating that the secondary condition was likely necessary, but not perhaps sufficient to produce hypercalcemia outside of excessive vitamin D supplementation from Soladek. Conversely, when the level of 25(OH) D is high but <200 ng/mL, it is advisable to seek another etiology as was the case in the series by Lowe et al. Hypercalcemia resolved in all patients after stopping the preparation [17].

It is commonly perceived that excessive sunlight exposure can be associated with vitamin D toxicity. However, studies have documented that full summer-long unprotected sun exposure cannot raise serum concentrations of 25(OH)D much more than 70–80 ng/mL. Whether one considers the changeable upper limits of normal for 25(OH)D at 50 or as high as 100 ng/mL, levels achieved by prolonged, unprotected sunlight exposure cannot by itself cause vitamin D toxicity (nl:

30–100) [18,19]. When dermal levels of vitamin D are high, vitamin D synthesis shunts to the production of inactive metabolites, which then can reenter the vitamin D synthesis pathway when dermal levels fall [20]. These recent observations help to document the widely held belief that sun alone cannot cause vitamin D toxicity. In situations where there is excessive conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D<sub>3</sub> such as in sarcoidosis or other granulomatous diseases; however, sun exposure can lead to hypercalcemia. While vitamin D is contained in fatty fishes (e.g., salmon, tuna) eggs, milk, and liver, the amounts are well under what would be needed to induce toxicity from these foods that naturally contain vitamin D. However, hypervitaminosis D has been associated with consumption of cow's milk when inadvertently fortified with massive concentrations of vitamin D. One investigation of eight patients manifesting symptoms of nausea, vomiting, weight loss, hyperirritability, or failure-to-thrive revealed markedly elevated mean concentrations of 25(OH)D of  $293 \pm 174$  ng/mL [4]. Analysis of the milk production facility at the local dairy revealed excessive vitamin D fortification of milk with up to 245,840 IU per liter (232,565 IU of vitamin D<sub>3</sub> per quart). Usual fortification of milk in the United States and Canada is 400 IU per quart. Milk is not fortified with vitamin D in most other parts of the world. In addition to milk, vitamin D fortification of natural foods includes plant-derived nondairy milks, breakfast cereals, yogurt, pasta, baked goods, fats, and orange juice [21]. Since the amount of vitamin D fortification is very modest, these foods are not generally suspected as a cause, bearing in mind of course the rare but important example of inadvertent toxic amounts of vitamin D fortification. In addition, industrial contamination of table sugar with vitamin D<sub>3</sub> and consequent severe vitamin D toxicity [25(OH)D level 623 ng/mL] has been reported [22].

Vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, although used interchangeably in the treatment of metabolic bone diseases, may differ in toxic potential at higher doses. A recent metanalysis of 24 studies suggests that cholecalciferol is more effective at increasing vitamin D levels than ergocalciferol [23]. Armas et al. [24] estimated the potency of vitamin D<sub>3</sub> to be three times that of D<sub>2</sub> when given in larger, intermittent doses. These data also suggested that D<sub>2</sub> has a shorter duration of action and that it accelerates the metabolism of D<sub>3</sub> in human subjects. The differential effects of D<sub>2</sub> versus D<sub>3</sub> may be of clinical significance only when larger doses are given intermittently. At doses of 50,000 IU per month, supplementation with D<sub>2</sub> results in lower serum 25(OH)D levels compared with D<sub>3</sub>; however, when given as 1600 IU/day, serum 25(OH)D levels are not different during supplementation with either D<sub>2</sub> or D<sub>3</sub> [25]. The current, officially recommended dietary allowance (RDA) for

vitamin D is 400 IU for infants 0–12 months of age, 600 IU for men and women from aged 1–70 years (including during pregnancy and lactation), and 800 IU for men and women over age 70 years [26]. These guidelines are controversial with many experts and authoritative bodies calling for increases in these recommended amounts [27–31]. Although this chapter concerns itself with vitamin D toxicity, the recommendation to increase nutritional amounts of vitamin D is due to the high prevalence of vitamin D deficiency worldwide (see Chapter 54) [32,33]. If the RDA for vitamin is to increase in the future, it is possible that the potential for vitamin D toxicity will increase.

While primary hyperparathyroidism in the developed world is typically associated with mild hypercalcemia with few, if any, symptoms, the clinical picture of PHPT in the developing world, in which vitamin D deficiency is more common, has tended to be more severe with skeletal and renal complications. However, a systematic review by Kumar Yadav et al. in 2020 found a significant trend toward a milder form of disease presentation in developing countries in recent years [34].

Vitamin D deficiency in the setting of PHPT is associated with a more severe phenotype [35], and many studies show larger adenoma size, higher serum parathyroid hormone and alkaline phosphatase levels, lower bone mineral density, and higher rates of fracture [36–40]. Moreover, patients with vitamin D deficiency are at greater risk to develop hypocalcemia due to “hungry bone” syndrome after parathyroidectomy. Patients with PHPT and vitamin D deficiency present a therapeutic dilemma due to the potential risk of worsening hypercalcemia with vitamin D supplementation. Gray et al. [41] treated 21 patients with mild PHPT (serum calcium  $10.8 \pm 0.5$  mg/dL) and coexistent vitamin D deficiency [25(OH)D < 20 ng/mL] with ergocalciferol 50,000 IU weekly for 4 weeks followed by 50,000 IU monthly for the remainder of a year. With a rise in 25(OH)D levels, PTH levels fell by 25%. The reduction in PTH levels by approximately 25% suggests that this component of the elevated PTH was due not to the endogenously overactive parathyroid gland but rather to the secondary hyperparathyroidism of the vitamin D deficiency state. Unlike most tissues, the parathyroid gland is able to take up 25(OH)D bound to vitamin D-binding protein (DBP) via its expression of the megalin/cubilin complex (see Chapter 7 and Chapter 17). Additionally, within the parathyroid gland, the 1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase) is very active and, thus, able to synthesize 1,25(OH)<sub>2</sub>D<sub>3</sub> from the substrate, 25(OH)D in the parathyroid gland. This action would directly suppress PTH production [20]. In the study by Gray et al. [41], the serum calcium increased from 10.5 to 11.9 mg/dL in one patient, and in another patient, the 24-hour urinary calcium excretion increased to over

400 mg/day. In the remaining patients, there were no substantial changes in serum calcium or urinary calcium excretion. Tucci [42] subsequently showed no overall increase in serum calcium or urinary calcium excretion with doses of ergocalciferol 50,000 IU weekly for 8 weeks among 56 patients with primary hyperparathyroidism. Grubbs et al. [43] also showed no change in serum calcium with a dosing regimen of 50,000 IU weekly or twice weekly for a mean duration of 4 weeks in patients with primary hyperparathyroidism. However, 3 of 56 patients, and 6 of 112 patients, respectively, did show increases in serum calcium during the follow-up period. At surgery in these patients, parathyroid adenoma size was smaller in patients who had achieved vitamin D sufficiency prior to parathyroidectomy.

Isidro et al. [44] studied 27 patients with PHPT-administered calcifediol [25(OH)D<sub>3</sub>] supplementation of up to 960 IU daily and found no increase in serum calcium levels. However, up to one-third of patients became hypercalciuric. A more recent randomized, placebo-controlled study in 46 subjects with PHPT [45] showed that doses of 2800 IU daily were safe and effective. Subjects were treated for 26 weeks prior to parathyroidectomy with 25(OH)D levels increasing from 50 to 94 nmol/L in the treatment group and remaining unchanged in the placebo group;  $P < .001$ . The vitamin D-supplemented group also showed a significant decrease in PTH and bone resorption markers and a significant increase in lumbar spine bone density. There were no statistical differences between groups with regard to serum or urine calcium excretion. Loh et al. conducted a metaanalysis to assess the effects of vitamin D replacement in 547 patients with mild primary hyperparathyroidism and coexistent vitamin D deficiency. With vitamin D supplementation, serum 25(OH)D levels improved with no worsening of hypercalcemia or hypercalciuria [46]. Song et al. included 11 articles with a total of 388 patients and reported similar findings [47].

These studies, in the aggregate, make two points. First, vitamin D supplementation in PHPT seems to be well tolerated and does not lead, in general, to worsening hypercalcemia or hypercalciuria. Second, the studies highlight the importance of careful monitoring. In particular, high doses of vitamin D over a short period of time are not to be recommended. Guidelines from the Fourth International Workshop on the Management of Asymptomatic Primary Hyperparathyroidism recommend supplementation of vitamin D starting at a dose of 800–1000 IU daily to achieve a minimum 25(OH)D level  $>20$  ng/mL [48]. The workshop participants noted that some experts recommend a goal of  $>30$  ng/mL in these patients.

The smallest dose of parent vitamin D (cholecalciferol or ergocalciferol) in healthy adults that can

produce toxicity and hypercalcemia is not known, but is clearly much higher than the RDA [26]. The current tolerable upper intake level (UL) recommended by the Food and Nutrition Board is 1000 IU daily for infants 0–6 months of age, 1500 IU daily for infants 7–12 months of age, 2500 IU daily for children 1–3 years of age, 3000 IU daily for children 4–8 years of age, and 4000 IU daily subsequently throughout life. Again, controversy exists regarding these guidelines [29–31]. Two small well-conducted clinical trials by Heaney et al. [49] and Barger-Lux et al. [2] showed that vitamin D<sub>3</sub> 10,000 IU daily for 8 and 20 weeks, respectively, did not cause an increase in serum calcium or any adverse effects in the combined cohort of 26 healthy men. In these subjects, mean 25(OH)D levels rose to 85 ng/mL ( $n = 10$ ) and 88 ng/mL ( $n = 16$ ) in the two studies, respectively. A review by Hathcock et al. [50] supports the selection of 10,000 IU daily as the UL based on these studies. Other studies of much shorter duration or concurrent treatment with prednisone and/or sodium fluoride have also shown that higher doses of vitamin D (up to 100,000 IU per day) were well tolerated. However, these vitamin D studies were not considered in their risk assessment due to short study lengths and potential confounders. In a randomized trial of calcium carbonate 1200 mg/day along with 10,000 IU vitamin D<sub>3</sub>/day (group 1) compared with the same dose of calcium plus 600 IU vitamin D<sub>3</sub>/day (group 2), the odds ratio for hypercalciuria was 3.6 in group 1 compared with group 2. However, the odds of developing hypercalcemia did not differ between the two groups [51].

Individuals manifest wide variations both in their response to hypercalcemic doses of vitamin D and in the duration of the effect. This variation in individual responsiveness might reflect differences in intestinal absorption and vitamin D metabolism, in the concentration of free vitamin D metabolites, in the rate of degradation of the metabolites and conversion to inactive metabolites, and in the capacity of storage sites for 25(OH)D [52]. Factors that enhance susceptibility to vitamin D toxicity and hypercalcemia include increased dietary calcium intake, reduced renal function, coadministration of vitamin A, presence of granulomatous disorders such as sarcoidosis, and genetic polymorphisms that render subjects more sensitive to vitamin D [4]. Hypercalciuria in hypervitaminosis D usually presents earlier than hypercalcemia, but it is easily missed for the obvious reason that urinary calcium is not routinely measured.

## 2.2 1,25-Dihydroxyvitamin D toxicity

The greater potency of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its direct actions on target tissues, in addition to its ability to inhibit

PTH synthesis and secretion, has made  $1,25(\text{OH})_2\text{D}_3$  and its analogs useful agents in patients with renal osteodystrophy and secondary hyperparathyroidism [53] (see Chapter 79).  $1,25(\text{OH})_2\text{D}_3$  is also used for treatment of patients with hypoparathyroidism, a rare disorder characterized by hypocalcemia and low or deficient PTH concentrations [54].  $1,25(\text{OH})_2\text{D}_3$  has also been found to inhibit the growth of human cancer cells in vitro [55]. As  $1,25(\text{OH})_2\text{D}_3$  is increasingly recognized for its antiproliferative, prodifferentiating, and immunomodulatory actions, its potential therapeutic use is expanding [56]. Mechanisms associated with the hypercalcemia due to  $1,25(\text{OH})_2\text{D}_3$  are increased intestinal absorption of calcium and potentiation of osteoclastic activity in bone. Dosages of  $1,25(\text{OH})_2\text{D}_3$  considerably above  $0.75\text{ }\mu\text{g/day}$  have been associated with toxicity, whereas dosages at or below  $0.5\text{ }\mu\text{g/day}$  rarely result in toxicity. One investigation showed that over 90% of patients on doses of  $1,25(\text{OH})_2\text{D}_3$  between  $1.0$  and  $2.0\text{ }\mu\text{g/day}$  became hypercalcemic, and all had hypercalciuria when calcium intake was set at  $1000\text{ mg per day}$  [57]. Accelerated deterioration of renal function was recorded in a number of reports in patients with renal insufficiency receiving  $1,25(\text{OH})_2\text{D}_3$  therapy [58]. Compared with oral therapy, intravenous administration of  $1,25(\text{OH})_2\text{D}_3$  to renal dialysis patients induces hypercalcemia less frequently, with a smaller increment in the serum calcium concentration and a more effective reduction of PTH levels [59]. Other studies, however, suggest that intermittent oral pulse administration of  $1,25(\text{OH})_2\text{D}_3$  may be effective, though not as effective as intravenous  $1,25(\text{OH})_2\text{D}_3$ , in suppressing PTH in uremic patients with secondary hyperparathyroidism [60–62] (see Chapter 79).

### 2.3 Toxicity due to synthetic vitamin D analogs

In one trial, oral pulse therapy with  $1\alpha$ -hydroxyvitamin  $\text{D}_3$  ( $1\alpha\text{-OHD}_3$ ) resulted in a rapid control of secondary hyperparathyroidism without causing hypercalcemia or hyperphosphatemia [63]. However,  $1\alpha\text{-OHD}_3$  harbors potential calcemic effects similar to  $1,25(\text{OH})_2\text{D}_3$  in the treatment of renal osteodystrophy. Crocker et al. [64] investigated the comparative toxicity of vitamin D,  $1\alpha\text{-OHD}_3$ , and  $1,25(\text{OH})_2\text{D}_3$  in weanling male mice at three different doses over a 4-week period.  $1\alpha\text{-OHD}_3$  appeared to be more toxic in the high-dose group only, with significantly higher serum calcium levels, higher urinary calcium excretion, and severe nephrocalcinosis.  $1\alpha\text{-OHD}_3$  has been described as less potent than  $1,25(\text{OH})_2\text{D}_3$  at low doses but equipotent at doses greater than  $2.0\text{ }\mu\text{g/day}$ . At the higher doses, there is a delayed onset of action and a prolonged half-life, suggesting a potential for cumulative toxicity in renal insufficiency [65,66].

The potential for hypercalcemia, hypercalciuria, and soft-tissue calcifications limits the clinical usefulness of  $1\alpha\text{-OHD}_3$ . Mortensen and colleagues compared the toxicity of both  $1\alpha\text{-OHD}_3$  and  $1,25(\text{OH})_2\text{D}_3$  in rats fed standard or low-calcium diets. High doses of either compound resulted in severe hypercalcemia, with retarded growth, nephrosis, and structural bone changes in the rats fed the standard diet. On the low-calcium diet, however, slight hypercalcemia occurred, but without growth retardation or bone changes. There was minimal effect on the kidney. Calcium restriction again proved effective in protecting the animals against the toxic effects of the vitamin D analogs. Animals fed the low-calcium diet tolerated  $1\alpha\text{-OHD}_3$  at dose levels up to 10 times higher than rats on the standard diets [67]. In human subjects,  $1\alpha\text{-OHD}_3$  can be associated with hypercalcemia at doses above  $1.0\text{ }\mu\text{g/day}$ , but amounts between  $0.5$  and  $1.0\text{ }\mu\text{g/day}$  appear to be safe.

Because of the relatively narrow therapeutic window of vitamin  $\text{D}_3$  1-hydroxylated compounds, a synthetic analog of vitamin  $\text{D}_2$ ,  $1\alpha\text{-OHD}_2$  (doxercalciferol), was developed with the concept that the window of therapeutic efficacy to toxicity would be wider. Doses of doxercalciferol ranging from  $1.0$  to  $5.0\text{ }\mu\text{g/day}$  were administered to 15 female postmenopausal osteopenic subjects. There was no evidence of vitamin D toxicity manifesting as either hypercalciuria or hypercalcemia, whereas significant therapeutic effects on osteoblastic activity were demonstrated [68]. Similar to the  $1\alpha\text{-OHD}_3$ , doxercalciferol requires obligatory hepatic 25-hydroxylation for activation. However, doxercalciferol is able to activate its catabolic pathway via hepatic 24-hydroxylation with a lower potential for toxicity [69].

Because of our emerging greater understanding of the nonclassic target tissue effects of vitamin D in the modulation of hormones and cytokines, and in the regulation of cellular differentiation and proliferation, newer clinical uses have been developed (see Sections X and XI of this book). The clinical applications of these newer properties of vitamin D, however, have also been tempered by the potential for complications, such as hypercalcemia and hypercalciuria. These concerns have prompted the development of additional analogs to better distinguish calcemic from antiproliferative effects [70] discussed in Section X of this book. Depending on the chemical modification of the basic structure of vitamin D, some analogs do demonstrate reduced calcemic activity, but others have been developed that exhibit increased calcemic activity due to enhanced intestinal calcium absorption and bone mineral mobilization. Fluorination of C-24, C-26, or C-27 apparently results in markedly increased calcemic activity resulting from reduced enzymatic



degradation of the side chain. Calcemic potency of  $1,25(\text{OH})_2\text{D}_3$  and its analogs can be also enhanced at least two- to fivefold by epimerization at the C-20 site [71].

The vitamin D analogs in use for secondary hyperparathyroidism in the United States include doxercalciferol and paricalcitol (19-nor-1,25-dihydroxyvitamin  $\text{D}_2$ ). Alfacalcidol, falecalcitriol, and 22-oxacalcitriol are in use outside the United States. Each analog retains suppressive action on PTH and parathyroid gland growth, but has less calcemic and phosphatemic activity than calcitriol. Paricalcitol and doxercalciferol appear to be equally effective in suppressing PTH; however, paricalcitol has been found to be less hypercalcemic [72]. Overall, the effect of vitamin D analogs to control the calcium–phosphate product might reduce vascular calcification and mortality in the renal failure population. However, observational and animal data [73,74] have yet to be confirmed by a randomized control trial [75]. Of additional potential importance may be the decreased likelihood of low bone turnover, or adynamic bone disease, with the use of these agents [76–78]. The mechanism for the differential actions of vitamin D analogs is not completely understood. Oxacalcitriol, for example, has a low affinity for vitamin D–binding protein, so more of the drug circulates in the free form, allowing it to be more rapidly metabolized than calcitriol [79]. This leads to a shorter half-life, which could explain the small and transient stimulation of intestinal calcium absorption. It does not, however, seem to account for the prolonged inhibition of PTH release. The use of analogs in end-stage renal disease is discussed in [Chapter 79](#).

Other vitamin D analogs, such as topical calcipotriol, have proved very effective in the treatment of psoriasis (see [Chapter 104](#)). Because of its low absorption rate and rapid degradation, calcipotriol is believed to have negligible effects on systemic calcium homeostasis when administered topically. However, isolated cases of hypercalcemia and hypercalciuria have been reported [80], even in patients using prescribed doses [81]. Bourke and colleagues noted suppression of serum PTH concentrations in all patients within 2 weeks of treatment with topical calcipotriol. Mean serum and urine calcium levels increased during treatment and fell following withdrawal [82]. The authors concluded that although this particular synthetic analog alters serum and urinary calcium with a dose-dependent effect on systemic calcium homeostasis, it is well tolerated and effective for mild to moderate chronic plaque psoriasis. However, it is potentially hazardous in extensive, unstable, exfoliative disease where increased absorption by inflamed and damaged skin can cause hypercalcemia [81].

### 3. Forms of endogenous Vitamin D toxicity

#### 3.1 Endogenous production of 25-hydroxyvitamin D

Endogenous dysregulation of vitamin D metabolites may be seen in Williams–Beuren syndrome (WBS; OMIM 194050), an idiopathic infantile form of hypercalcemia. WBS is also associated with elfin facies, late psychomotor development, selective mental deficiency, and supravalvular aortic stenosis [83]. Among 232 individuals with WBS aged 0–67 years, the prevalence of hypercalcemia was 17% in infants and 26% in toddlers [84]. The level of hypercalcemia has been reported to range widely from 12 to 19 mg/dL and usually subsides by 4 years of age; however, it may recur during puberty. The etiology of the hypercalcemia in WBS remains unknown. Early reports suggested an exaggerated production of  $25(\text{OH})\text{D}$  with small doses of vitamin D as a possible cause of the hypervitaminosis D [85]. However, recent cases have shown normal  $25(\text{OH})\text{D}$  levels, which may indicate an etiology unrelated to abnormal vitamin D metabolism [86,87].

#### 3.2 Endogenous production of 1,25-dihydroxyvitamin D

##### 3.2.1 Granulomatous diseases

In contrast to the megadosages of exogenous vitamin D that are usually required to produce vitamin D toxicity, patients with granulomatous diseases can develop hypercalcemia rather easily without excessive intake of exogenous vitamin D. They are said to be hypersensitive to vitamin D. The etiology of the vitamin D toxicity in this syndrome is due to poorly regulated extrarenal synthesis of  $1,25(\text{OH})_2\text{D}_3$  by the granulomatous tissue itself. In contrast to the various presentations of vitamin D toxicity described earlier, the responsible metabolite in granulomatous disease is quite different. In the case of vitamin D toxicity due to overdosage of vitamin D or  $25(\text{OH})\text{D}$ ,  $25(\text{OH})\text{D}$  becomes the active metabolite; renal production of  $1,25(\text{OH})_2\text{D}_3$  in this setting is highly regulated and not excessively high due to suppression of PTH by the slightest rise in serum calcium and feedback inhibition of  $1\alpha\text{-OHase}$  by the rising levels of  $1,25(\text{OH})_2\text{D}_3$ . In granulomatous tissue, however,  $1,25(\text{OH})_2\text{D}_3$  formation is not subject to control by any recognized regulators, such as PTH, phosphorus, or calcium. Thus, this syndrome is due to ectopic production of  $1,25(\text{OH})_2\text{D}_3$  by the granulomatous tissue itself. The mechanisms by which hypercalcemia occurs, however, are similar to all other vitamin D toxic states, namely, increased intestinal calcium absorption and enhanced osteoclastic

bone resorption [88,89]. Many studies have led to greater understanding of the pathophysiology and immunological features associated with this syndrome. The role of extrarenal  $1\alpha$ -OHase activity as a source of endogenous  $1,25(\text{OH})_2\text{D}_3$  production in granulomatous disease and other disorders is discussed in much greater detail in [Chapter 9](#).

### 3.2.2 Sarcoidosis

Abnormalities in calcium metabolism have long been noted in patients with sarcoidosis [90]. Sarcoidosis is also the most common granulomatous disease associated with hypercalcemia. Approximately 10%–20% of patients with sarcoidosis may develop hypercalcemia [91,92]. However, severe hypercalcemia is uncommon [93]. Approximately 50% of patients will experience hypercalciuria at some time during the course of the disease, with hypercalciuria invariably present when patients develop hypercalcemia [77]. In the 1950s, studies had already revealed similarities between hypercalcemia of sarcoidosis and the hypercalcemia of vitamin D toxicity, namely, increased intestinal absorption of calcium, hypercalciuria, and therapeutic efficacy of glucocorticoids [94,95]. The major distinguishing feature was in the amount of vitamin D associated with the hypercalcemia and/or hypercalciuria. Seasonal variation of the serum calcium level in sarcoidosis was correlated with availability of sunlight as a source of vitamin D [96]. In the late 1970s, two independent groups showed that the vitamin D–like principle that appeared to be responsible in sarcoidosis was, in fact, the active metabolite of vitamin D,  $1,25(\text{OH})_2\text{D}_3$  [89,97].

Ectopic production of  $1,25(\text{OH})_2\text{D}_3$  was confirmed by demonstrating high circulating concentrations of  $1,25(\text{OH})_2\text{D}_3$  in anephric patients with sarcoidosis on hemodialysis during hypercalcemic episodes [98,99]. This observation showed unequivocally that the kidney, usually the predominant source of  $1,25(\text{OH})_2\text{D}_3$  in nonpregnant individuals, could not be the source of  $1,25(\text{OH})_2\text{D}_3$  in these patients. The serum calcium and  $1,25(\text{OH})_2\text{D}_3$  levels were positively correlated with indices of disease activity [100–102], namely, the extent of granuloma formation and the angiotensin-converting enzyme level. It was subsequently shown that the granulomatous tissue was, in fact, the site of  $1,25(\text{OH})_2\text{D}_3$  production. The  $1\alpha$ -hydroxylase enzyme responsible for formation of  $1,25(\text{OH})_2\text{D}_3$  was present in lymph node homogenates [103]. Moreover, pulmonary alveolar macrophages [104] could be shown to catalyze the formation of an  $^3\text{H}$ -labeled  $25(\text{OH})\text{D}_3$  metabolite. This metabolite was definitively identified as  $1,25(\text{OH})_2\text{D}_3$  by high-performance liquid chromatography (HPLC), by the chick intestinal receptor assay for  $1,25(\text{OH})_2\text{D}_3$ , by UV spectroscopy, and by mass

spectrometry [105]. The production of mRNA for  $1\alpha$ -OHase (CYP27B1) is markedly increased in alveolar macrophages isolated from hypercalcemic patients with sarcoid [106]. Importantly, control of the macrophage  $1\alpha$ -OHase enzyme differs from that of the renal  $1\alpha$ -OHase. The renal  $1\alpha$ -OHase is regulated at the level of transcription by calcitropic hormones, especially PTH, and is exquisitely autoregulated by  $1,25(\text{OH})_2\text{D}_3$  itself [107]. In contrast, macrophage  $1\alpha$ -OHase mRNA expression is potently stimulated by inflammatory agents, such as  $\gamma$ -interferon [108], and shows no feedback control in response to  $1,25(\text{OH})_2\text{D}_3$  [109]. Communication between signaling pathways of  $\gamma$ -interferon and the vitamin D receptor has been reported [110]. These mechanisms account for the uncontrolled synthesis of  $1,25(\text{OH})_2\text{D}_3$  and the characteristic finding of increased sensitivity to vitamin D in these patients [111,112]. Another property of the macrophage  $1\alpha$ -OHase enzyme is that it is inhibited in a dose-dependent fashion by dexamethasone and chloroquine that do not influence the renal  $1\alpha$ -OHase enzyme that catalyzes synthesis of  $1,25(\text{OH})_2\text{D}_3$  [113]. These in vitro observations have direct clinical relevance and suggest possible treatments of hypercalcemia due to sarcoidosis. There are several mechanisms by which calcium metabolism is disturbed in sarcoidosis [114]. First,  $1,25(\text{OH})_2\text{D}_3$  causes hypercalcemia, in part, by stimulating intestinal calcium absorption. A low-calcium diet [115,116], alone or in association with cellulose phosphate [117], was found to normalize the calcium level in some patients with sarcoidosis. Second,  $1,25(\text{OH})_2\text{D}_3$  directly stimulates osteoclastic-mediated bone resorption; skeletal granulomas are not required for this effect [118–120]. The increased flux of calcium into the extracellular space by these gastrointestinal and skeletal mechanisms, aided by suppression of PTH [97–99], leads to hypercalciuria. Chronic hypercalciuria favors nephrocalcinosis and renal stone formation [121]. When the kidneys are unable to excrete the calcium presented to them, because of either declining renal function, enhanced bone resorption, a sudden influx of dietary calcium, dehydration, or any combination of these events, hypercalcemia ensues [122]. Granulomatous production of PTHrP may also play a role in abnormal calcium metabolism, where TNF $\alpha$  and interleukin-6, produced by macrophages, increase PTHrP gene expression. For example, a 67-year-old man with sarcoidosis developed hypercalcemia with concurrently elevated PTHrP. Immunohistochemistry staining demonstrated PTHrP expression in bone marrow granulomas. Treatment with glucocorticoids normalized hypercalcemia and the PTHrP level [123]. In another series, PTHrP was present in 85% of biopsies of granulomatous tissue from patients with sarcoidosis [124].

### 3.2.3 Tuberculosis

Longitudinal studies from the United States [125] and India [126] suggested that 16%–28% of patients with tuberculosis develop hypercalcemia. However, in these early studies, vitamin D supplements were employed, increasing the risk and severity of hypercalcemia. A similar study from Greece [127] reported a figure as high as 48% when serum calcium was corrected to a normal albumin level. Other studies from the United Kingdom [128], Belgium [129], Hong Kong [130], and Malaysia [131] have shown a much lower prevalence of hypercalcemia, in the range of 0%–2.3%. It is likely that hypercalcemia is not as common in tuberculosis as was previously thought [132]. This discrepancy might be attributable to regional differences in calcium and vitamin D intake, which can unmask hypercalcemia [133], along with increased sun exposure.

Reports of high circulating levels of  $1,25(\text{OH})_2\text{D}_3$  in three anephric patients with tuberculosis support an extrarenal source of the active vitamin D metabolite [134,135]. Positive correlation of the albumin-adjusted calcium level with the radiographic extent of the disease has been shown [130]. Hypercalcemia in tuberculosis may occur weeks to months after starting antituberculosis chemotherapy [125,126]. Thus, the hypercalcemia is not related to the presence of viable acid-fast bacilli, but rather to the granulomatous process and associated reactions. As with sarcoidosis, hypercalcemia in tuberculosis can be controlled by administration of glucocorticoids [136].

In patients with tuberculous pleuritis, the mean free  $1,25(\text{OH})_2\text{D}_3$  concentration in pleural fluid was selectively concentrated by 5.3-fold over that in serum [137]. Positive correlation between the concentrations of substrate  $25(\text{OH})\text{D}_3$  and product  $1,25(\text{OH})_2\text{D}_3$  in pleural fluid supported the idea that  $1,25(\text{OH})_2\text{D}_3$  was produced locally by activated inflammatory cells in or adjacent to the pleural space. The pleural fluid was found to have high concentrations of  $\gamma$ -interferon, a cytokine known to stimulate activated macrophages in vitro to synthesize  $1,25(\text{OH})_2\text{D}_3$  [138]. Cells obtained from bronchoalveolar lavage in patients with tuberculosis were also found to synthesize  $1,25(\text{OH})_2\text{D}_3$  in vitro. An important source of the active vitamin D metabolite appears to be the CD8+ T lymphocytes at the granulomatous sites [139]. If one considers etiologically about the production of  $1,25(\text{OH})_2\text{D}_3$  under these circumstances, the immunomodulatory functions of  $1,25(\text{OH})_2\text{D}_3$  acting as a beneficial local paracrine factor could be pertinent. In fact, increased levels of  $1,25(\text{OH})_2\text{D}_3$  have been found to result in the production of cathelicidin, a peptide with antimicrobial properties against *Mycobacterium tuberculosis*, by macrophages [140] (see Chapter 94 and Chapter 98). Viewed in this

context, hypercalcemia occurs when  $1,25(\text{OH})_2\text{D}_3$  is produced in such quantities that it gains entry into the circulation. Hypercalcemia in tuberculosis is usually mild and asymptomatic. Besides glucocorticoids, ketoconazole administration has been associated with a rapid decline in  $1,25(\text{OH})_2\text{D}_3$  and normalization of serum calcium levels, through inhibition of  $1\alpha$ -OHase [141]. Long-term antituberculosis therapy with isoniazid and rifampin can also be effective in treating the hypercalcemia by controlling the disease.

### 3.2.4 Foreign material

Introduction of foreign material into the human body can lead to a granulomatous inflammatory reaction resulting in hypercalcemia due to excessive  $1,25(\text{OH})_2\text{D}_3$  production. A growing number of substances have been reported to induce this reaction including talc-induced granulomatous pneumoconiosis pneumonia [142], granulomatous reaction to silicone injections [143–146], polymethylmethacrylate injections [147–149], and cosmetic injections of paraffin oil [150] and mineral oil [151]. Particle disease, resulting from an inflammatory macrophage reaction to shredded particulates from a failed prosthetic joint, has also been associated with hypercalcemia and elevated  $1,25(\text{OH})_2\text{D}_3$ , which was responsive to treatment with glucocorticoids [152].

### 3.2.5 Other granulomatous diseases

Hypercalcemia associated with other granulomatous disorders is relatively rare and the subject of case reports, but it has been described in infectious etiologies including coccidioidomycosis [153], histoplasmosis [154], candidiasis [155], cat-scratch disease [156], *Pneumocystis jirovecii* pneumonia [157–161], paracoccidioidomycosis [162], and *Mycobacterium avium* complex [163]. Immune reconstitution inflammatory syndrome led to hypercalcemia with elevated  $1,25(\text{OH})_2\text{D}_3$  in three patients with a documented history of HIV who also had mycobacterium tuberculosis [164,165] or *Mycobacterium avium* complex [166].

Hypercalcemia was also reported as a systemic reaction to Calmette-Guérin bacillus (BCG) therapy for bladder cancer complicated by disseminated granulomatosis [167]. Noninfectious etiologies, aside from sarcoidosis, have been reported with granulomatosis with polyangiitis [168–170], Langerhans' cell granulomatosis [171], Crohn's disease [172–174], infantile fat necrosis [175], giant cell polymyositis [176], gastrointestinal stromal tumors [177–179], and in some patients on chronic hemodialysis with hepatic granulomatosis of unknown etiology [180].

Malakoplakia, due to nongranulomatous macrophage infiltration, has also been associated with



hypercalcemia due to 1- $\alpha$ -hydroxylase activity in the malakoplakia cell [181]. Massive lymphadenopathy resulting from proliferation of histiocytes in lymph nodes, an entity known as Rosai-Dorfman disease, led to hypercalcemia and increased serum 1,25(OH) $_2$ D $_3$  levels in one case [182]. Acute granulomatous pneumonitis, a rare complication of methotrexate therapy, can also lead to hypercalcemia with elevated 1,25(OH) $_2$ D $_3$  [183].

Several rare dermatologic conditions including necrobiotic xanthogranuloma, a chronic granulomatous disease primarily involving the skin [184], palisaded neutrophilic and granulomatous dermatitis, a benign inflammatory dermatosis [185], and granulomatous slack skin disease, a rare T cell lymphoproliferative disorder, have all been associated with hypercalcemia and elevated 1,25(OH) $_2$ D $_3$  in Ref. [186]. Additionally, idiopathic hypereosinophilic syndrome has also been shown to cause hypercalcemia with elevated 1,25(OH) $_2$ D $_3$  [187].

The mechanism of increased production of the active vitamin D metabolite is believed to be shared by all of these granulomatous disorders. Autonomous excess production of calcitriol in the absence of granulomatous disease has also been reported in patients with elevated angiotensin converting enzyme levels [188], presumably with increased calcitriol production by macrophages. A possible role for 1,25(OH) $_2$ D $_3$  in these granulomatous disorders as noted before includes immunomodulatory features. Interesting, an experimental analog of cAMP (8-Cl-cAMP) given to patients with advanced solid malignancies was shown to cause hypercalcemia associated with elevated levels of 1,25(OH) $_2$ D $_3$  not clearly associated with a granulomatous reaction [189].

### 3.2.6 Lymphoma

Hypercalcemia has been reported to occur in up to 5% [190] and 15% [191] of patients with Hodgkin's disease and non-Hodgkin's lymphoma (NHL), respectively. Up to 80% of patients with human T cell leukemia virus type 1 (HTLV-1)–associated adult T cell lymphoma/leukemia (ATLL) may develop hypercalcemia [192]. As is the case with other malignancies, hypercalcemia is a poor prognostic feature in lymphoma [193,194], adding substantially to morbidity and mortality. The humoral mediators of hypercalcemia in lymphoma are multiple and heterogenous. However, evidence has shown 1,25(OH) $_2$ D $_3$  to be an important factor in many cases.

The development of hypercalcemia in Hodgkin's disease is most consistently associated with 1,25(OH) $_2$ D $_3$ . Since the first report of hypercalcemia complicating Hodgkin's disease in 1956 [195], more than 60 cases have been described. In a retrospective review of the literature [196], 84% of patients had a peak serum calcium above 12 mg/dL, 74% of the patients had Ann

Arbor stage III or IV disease, and 68% were symptomatic with night sweats, fever, and weight loss. Only 3 of 23 patients had radiological evidence of lytic bone lesions. In 17 hypercalcemic patients, all but one patient had an elevated 1,25(OH) $_2$ D $_3$  level. There is no evidence to implicate parathyroid hormone–related peptide (PTHrP) as a mediator of hypercalcemia in Hodgkin's disease. Two patients with Hodgkin's disease [197,198] were reported to have intermittent hypercalcemia during two consecutive summers or on vitamin D challenge. There was a close association between hypercalcemia and the high 1,25(OH) $_2$ D $_3$  level, with the serum 25(OH)D $_3$  being maintained within the normal range. These observations support the idea that the mechanism of the hypercalcemia in Hodgkin's disease is similar to that of the granulomatous diseases, namely, production by the lymphomatous tissue of 1,25(OH) $_2$ D $_3$ .

A number of cases of 1,25(OH) $_2$ D $_3$ -induced hypercalcemia in non-Hodgkin's lymphoma have been described [196]. Most patients had bulky or advanced-stage disease, but no clinically or radiographically evident bone lesions. In one case, the 1,25(OH) $_2$ D $_3$ -mediated hypercalcemia was associated with transformation from a chronic lymphocytic leukemia to an aggressive high-grade non-Hodgkin's lymphoma [199]. Seymour et al. [200] prospectively studied patients with non-Hodgkin's lymphoma and found that 12 of 22 patients (55%) with hypercalcemia had elevated serum calcitriol levels compared with a group of patients with hypercalcemia due to multiple myeloma. Additionally, 71% of patients with normal serum calcium had hypercalciuria, and 18% had elevated serum 1,25(OH) $_2$ D $_3$  levels. Data supporting extrarenal synthesis of 1,25(OH) $_2$ D $_3$  include the presence of severe renal failure in a number of instances [201,202]; the demonstration of in vitro conversion of 25(OH)D $_3$  to 1,25(OH) $_2$ D $_3$  by excised lymph node homogenates [203]; the prompt decline of 1,25(OH) $_2$ D $_3$  levels to normal after excision of an isolated splenic lymphoma [204]; a primary ovarian lymphoma [205]; and sensitivity to glucocorticoid suppression [202]. 5 of 10 patients with either HIV or non-HIV-associated non-Hodgkin's lymphoma and hypercalcemia had frankly elevated serum 1,25(OH) $_2$ D $_3$  concentrations [206]. Other malignant lymphoproliferative diseases associated with 1,25(OH) $_2$ D $_3$ -mediated hypercalcemia include lymphomatoid granulomatosis [207], dysgerminoma [208], and an inflammatory myofibroblastic tumor [209].

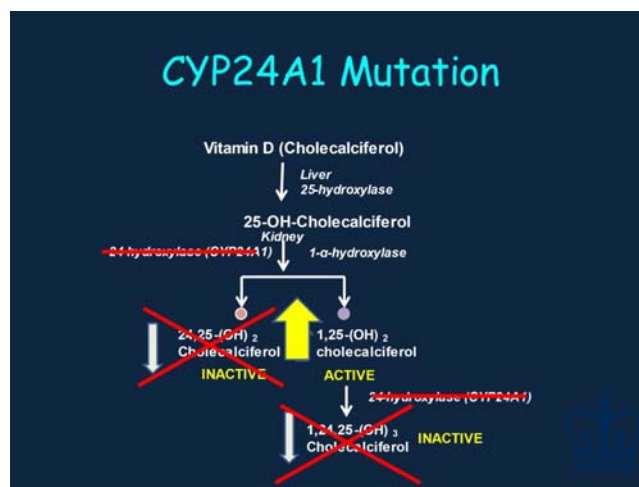
The precise cell type responsible for the extrarenal synthesis of 1,25(OH) $_2$ D $_3$  in lymphoma remains to be established. There are two possibilities. One is the tumor-infiltrating reactive macrophage, recognized by a "starry-sky" appearance [210] in intermediate and high-grade lymphomas, in which hypercalcemia is also



Although HTLV-1-transformed lymphocytes were shown *in vitro* to possess the capacity to convert 25(OH)D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub> [217], most studies have shown reduced 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in hypercalcemia associated with HTLV-1-related adult T cell leukemia/lymphoma [218,219]. PTHrP is most strongly implicated as the major mediator in this syndrome [220]. PTHrP messenger RNA has been demonstrated in HTLV-1-infected T cells [221] and tumor cells from adult T cell lymphoma/leukemia (ATLL) patients with hypercalcemia [222]. Nevertheless, there are two well-documented instances of elevated 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in ATLL [192,201]. In the first case, a PTHrP level was not available. In the second case, concomitant elevation of 1,25(OH)<sub>2</sub>D<sub>3</sub> and PTHrP was shown, suggesting the possibility of increased renal 1 $\alpha$ -OHase activity secondary to PTHrP. Alternatively, the tissue could be the site of both PTHrP and 1,25(OH)<sub>2</sub>D<sub>3</sub> formation. Other malignancies associated with 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated hypercalcemia include seminoma [223], leiomyoblastoma [224], and squamous cell bronchogenic carcinoma [225]. Most patients with hypercalcemia due to classic squamous cell carcinoma have elevated PTHrP levels and either suppressed or normal 1,25(OH)<sub>2</sub>D<sub>3</sub> levels [226].

The 24-hydroxylase (24-OHase) enzyme is responsible for the metabolism of active 1,25(OH)<sub>2</sub>D<sub>3</sub> to the inactive metabolite 1,24,25-trihydroxyvitamin D (1,24,25(OH)<sub>2</sub>D<sub>3</sub>). Loss of 24-OHase, the enzyme produced by the *CYP24A1* gene, prevents inactivation of vitamin D metabolites at several steps (Fig. 81.1). Inactivating mutations in *CYP24A1* gene can result in idiopathic infantile hypercalcemia (OMIM 143880), characterized by hypercalcemia, hypotonia, nephrocalcinosis and failure to thrive in infancy. Schlingmann et al. reported six patients from four families with idiopathic infantile hypercalcemia and found loss-of-function mutations in *CYP24A1* that appeared to be associated with disease development [227]. The disease has also been diagnosed in adults. Tebben et al. reported a family of patients with hypercalcemia, hypercalciuria, nephrolithiasis, and elevated 1,25-dihydroxyvitamin D levels [228]. Genetic testing revealed *CYP24A1* mutations in three generations of this family. A report from Jacobs et al. described a man with a long-standing history of hypercalcemia and nephrolithiasis diagnosed in his early 20s who was eventually diagnosed with an inactivating mutation of *CYP24A1* at age 73 years after the advent of genetic testing [229]. There have been multiple subsequent case reports in adults diagnosed with this disorder [228,230–236].

Vitamin D toxicity may occur in patients due to any one of the three forms of vitamin D, namely, the



**FIGURE 81.1** The effects of inactivating mutations in the CYP24A1 gene with resultant loss of 24-hydroxylase activity on the catabolism of vitamin D metabolites. *Figure adapted with permission from Marcella Walker, MD.*

vitamin D parent compound, 25(OH)D or 1,25(OH)<sub>2</sub>D<sub>3</sub>. Multiple factors may influence susceptibility to vitamin D toxicity and include the concentration of the vitamin D metabolite itself, vitamin D receptor (VDR) number, activity of 1 $\alpha$ -OHase, the metabolic degradation pathway, and the capacity of the DBP. Vitamin D<sub>2</sub> or D<sub>3</sub> toxicity is more difficult to manage than toxicity due to its metabolites 25(OH)D or 1,25(OH)<sub>2</sub>D<sub>3</sub>. In part, this is due to the extensive lipid solubility of the parent compound in liver, muscle, and fat tissues and corresponding large storage capacity. As a result, the half-life of vitamin D ranges from 20 days to months. In contrast, the biological half-life of the less lipophilic compound 25(OH)D is shorter, approximately 15 days [237]. The biological half-life of the least lipophilic compound 1,25(OH)<sub>2</sub>D<sub>3</sub> is much shorter, approximately 15 h [238]. In general, duration of toxicity is related to the half-life of the vitamin D compound. Thus, the hypercalcemia of parent vitamin D overdose can theoretically last for as long as 18 months, long after dosing is discontinued, because of its slow release from fat deposits. Overdosage of 25(OH)D can persist for weeks also, but excessive 1,25(OH)<sub>2</sub>D<sub>3</sub> toxicity is more rapidly reversed because 1,25(OH)<sub>2</sub>D<sub>3</sub> is not stored in appreciable amounts in the body [101].

The toxicity of either parent vitamin D or 25(OH)D is due to 25(OH)D. In an investigation examining the concentrations of vitamin D<sub>3</sub> and its metabolites in the rat as influenced by various intakes of vitamin D<sub>3</sub> or 25(OH)D, Shepard and DeLuca found that intakes of vitamin D<sub>3</sub>, ranging from 1 to 10,000 IU daily (0.65–6500 nmol/day), resulted in excessive concentrations of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> but not in 1,25(OH)<sub>2</sub>D<sub>3</sub> [239]. Similarly, increased dosages of 25(OH)D<sub>3</sub> ranging from 0.46 to 4600 nmol/day resulted in excessive amounts of 25(OH)D<sub>3</sub>, but not of vitamin D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub>. Unlike 1,25(OH)<sub>2</sub>D<sub>3</sub> whose production is tightly regulated in the kidney, the production of 25(OH)D in the liver is not tightly controlled. The high capacity for 25-hydroxylation of vitamin D in the liver as well as limited regulation at this site allows for massive amounts of 25(OH)D to be generated from large amounts of vitamin D. Thus, excessive concentrations of 25(OH)D are typically measured in vitamin D toxicity. Hypercalcemia appears to result only when 25(OH)D concentrations are consistently above 150 or 200 ng/mL (375 nmol/L) in normal individuals [3,240]. In patients with a secondary condition that can contribute to the development of hypercalcemia, toxicity can develop at lower 25(OH)D concentrations [17]. As would be expected, PTH levels are suppressed in this form of hypercalcemia. In the setting of toxicity due to overadministration of 1,25(OH)<sub>2</sub>D<sub>3</sub>, the active metabolite itself is responsible for the hypercalcemia [241].

## 4.2 Role of vitamin D receptor in vitamin D toxicity

Various investigations have helped to shed light on the interrelationship among vitamin D metabolites, the VDR, and PTH in vitamin D toxicity. The biologically active form of vitamin D, 1,25(OH)<sub>2</sub>D<sub>3</sub>, as is typical of other steroid hormones, binds to a specific intracellular receptor protein (VDR) within its target tissues. The hormone–VDR complex then triggers subsequent transcriptional events by binding to DNA elements.

Regulation of cellular VDR numbers is believed to be an important mechanism by which cellular responsiveness to 1,25(OH)<sub>2</sub>D<sub>3</sub> is modulated, because the biological activity of 1,25(OH)<sub>2</sub>D<sub>3</sub> is proportional both to tissue VDR number and concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Increased VDR concentrations imply enhanced tissue responsiveness to 1,25(OH)<sub>2</sub>D<sub>3</sub>, whereas decreased receptor numbers indicate reduced tissue responsiveness. Several investigations have suggested that exogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> can lead to homologous upregulation of VDR in vitro and in vivo, in contrast to endogenous production of 1,25(OH)<sub>2</sub>D<sub>3</sub>. In vitro and in vivo administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> to rats has been shown to increase VDR content. In vitro exposure of human skin fibroblasts and osteosarcoma cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to result in a three- to fivefold increase in VDR number [242]. Similarly, in vivo studies have shown increased VDR with exogenous administration of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Costa and Feldman administered 1500 pmol/kg of 1,25(OH)<sub>2</sub>D<sub>3</sub> daily to rats and found a 30% increase in intestinal VDR and a threefold increase in renal VDR concentration [243]. Reinhart et al. infused rats with 250 pmol/kg of 1,25(OH)<sub>2</sub>D<sub>3</sub> daily for 6 days and noted a 22% increase in VDR levels in the intestine and a 37% increase in bone [244]. Goff and colleagues infused 36 ng of 1,25(OH)<sub>2</sub>D<sub>3</sub> to rats over 7 days and found a 1.5-fold increase in duodenal VDR content and a threefold increase in renal VDR content [245].

Goff et al. [245] also demonstrated that endogenously produced 1,25(OH)<sub>2</sub>D<sub>3</sub> has a different effect than exogenous administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> on tissue VDR content. Rats fed a calcium-restricted diet resulting in “nutritional” hyperparathyroidism achieved a similar increase in endogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration as rats administered exogenous 1,25(OH)<sub>2</sub>D<sub>3</sub>. However, calcium-restricted rats failed to upregulate VDR content in the duodenum or kidney, presumably a consequence of the negative control of VDR by PTH [246]. This point has at least conceptual relevance in the case of vitamin D toxicity. Rather than downregulation occurring during hypervitaminosis D, which is a more typical regulatory and protective event to limit tissue responsiveness, exposure of cells to exogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> results in enhanced responsiveness by virtue of upregulation.

Such a mechanism would be of particular clinical relevance if the toxicity were due to overexposure of  $1,25(\text{OH})_2\text{D}_3$ . Moreover, in this setting, the associated suppression of PTH would prevent the regulatory mechanism from being operative.

Evidence suggests that in parent vitamin D toxicity, target tissues are responding to high concentrations of  $25(\text{OH})\text{D}$ , not  $1,25(\text{OH})_2\text{D}_3$ . Concentrations of  $1,25(\text{OH})_2\text{D}_3$  are typically only slightly increased, if at all. The hypercalcemia is due to the effects of pharmacologically high levels of  $25(\text{OH})\text{D}$ , even though  $25(\text{OH})\text{D}$  has a much lower binding affinity for the VDR than  $1,25(\text{OH})_2\text{D}_3$ . At high concentrations,  $25(\text{OH})\text{D}$  can compete for binding at VDR sites and thereby produce biological effects similar to those of  $1,25(\text{OH})_2\text{D}_3$  on intestine and bone [247]. Beckman and colleagues [248] suggested, furthermore, that hypervitaminosis D, like excessive exogenous  $1,25(\text{OH})_2\text{D}_3$ , is associated with homologous upregulation of intestinal VDR. Their investigation demonstrated that supraphysiological amounts of vitamin  $\text{D}_2$  or vitamin  $\text{D}_3$  administered to rats at doses of 25,000 IU daily for 6 days resulted in increasing plasma  $25(\text{OH})\text{D}$  concentrations with significant upregulation of intestinal VDR concentration and hypercalcemia. Plasma  $1,25(\text{OH})_2\text{D}_3$  levels were not altered substantially.

A comparison between hypervitaminosis  $\text{D}_3$  and  $\text{D}_2$  was also made [248]. No differences in  $25(\text{OH})\text{D}$  and plasma calcium concentrations were noted between either of the preparations. Concentrations of  $25(\text{OH})\text{D}$  in each case were markedly higher than the control group. The concentration of  $1,25(\text{OH})_2\text{D}_3$  was observed to be only slightly greater in the vitamin  $\text{D}_3$ -treated group than the vitamin  $\text{D}_2$ -treated group. Because the  $25(\text{OH})\text{D}$  concentrations were elevated 20- to 25-fold, whereas  $1,25(\text{OH})_2\text{D}_3$  showed only minimal increases, the biochemical and clinical changes associated with parent vitamin D toxicity were attributed to  $25(\text{OH})\text{D}$ . The data provided further support for the importance of  $25(\text{OH})\text{D}$  as the major toxic metabolite in vitamin D-associated hypercalcemia, as well as for the importance of increased intestinal VDR in the pathophysiological process that leads to enhanced effects of this metabolite.

### 4.3 Control of renal $1\alpha$ -OHase in vitamin D toxicity

Some investigators have suggested that toxic effects of excessive concentrations of  $25(\text{OH})\text{D}$  may result from PTH suppression and downregulation of  $1\alpha$ -OHase with increased concentrations of  $25(\text{OH})\text{D}$ . PTH and  $1,25(\text{OH})_2\text{D}_3$  have known reciprocal actions on  $1\alpha$ -OHase and  $24$ -OHase activities. PTH stimulates

$1\alpha$ -OHase activity and downregulates  $24$ -OHase activity;  $1,25(\text{OH})_2\text{D}_3$ , on the other hand, downregulates  $1\alpha$ -OHase activity and stimulates  $24$ -OHase activity.

Beckman and colleagues [249] studied the effects of an excess of vitamin  $\text{D}_3$  and dietary calcium restriction on tissue  $1\alpha$ -OHase and  $24$ -OHase activity in rats. Four groups of rats with different dietary calcium and vitamin  $\text{D}_3$  concentrations were studied (normal calcium, NC; low calcium, LC; and the excess vitamin D groups with normal or low calcium, NCT and LCT). The data showed that in the setting of a calcium-restricted diet, a nutritional hyperparathyroidism ensued. Under conditions of excess vitamin  $\text{D}_3$  at doses of 75,000 IU per week and on a calcium-restricted diet, elevations in PTH facilitated the elimination of  $25(\text{OH})\text{D}_3$  through its metabolism to  $1,25(\text{OH})_2\text{D}_3$  and/or degradation to  $24,25(\text{OH})_2\text{D}_3$ . The elevation in PTH was accompanied by increased activation of renal  $1\alpha$ -OHase activity, lower concentrations of  $25(\text{OH})\text{D}_3$ , increased activation of intestinal  $24$ -OHase activity, and lower renal VDR content compared with the normal calcium group. In contrast, the normal calcium diet in the vitamin  $\text{D}_3$  excess group contributed to the toxicity by virtue of suppressed PTH concentrations resulting in downregulation of renal  $1\alpha$ -OHase and decreased  $24$ -OHase activity, and, thus, higher  $25(\text{OH})\text{D}_3$  concentrations. On the other hand, dietary calcium restriction in the setting of vitamin  $\text{D}_3$  excess seemed to be protective, providing less biological stimulation due to higher PTH concentrations with reduced VDR, increased activation of both  $1\alpha$ -OHase and  $24$ -OHase activities, greater reductions in  $25(\text{OH})\text{D}_3$  concentrations, and lower concentrations of total calcium resulting in a less toxic state. So the low-calcium diet protects, not only by contributing to less hypercalcemia, but also by facilitating metabolic pathways of vitamin D inactivation.

### 4.4 Inhibition of the catabolic pathway of $24$ -OHase

Inhibition of the enzymes that degrade the vitamin D metabolites has a role in the pathogenesis of hypervitaminosis D.  $1,25(\text{OH})_2\text{D}_3$  is a known regulator of its own catabolism and an inhibitor of its synthesis. In the kidney, intestine, and other targets,  $1,25(\text{OH})_2\text{D}_3$  induces the enzyme  $24$ -OHase. This enzyme initiates a catabolic cascade that ultimately causes side chain oxidation, cleavage, and metabolic elimination of both  $1,25(\text{OH})_2\text{D}_3$  and  $25(\text{OH})\text{D}$ . It accounts for 35%–40% of the catabolism of  $1,25(\text{OH})_2\text{D}_3$  [250]. The remainder of the metabolic degradation is due to other side chain oxidations and biliary clearance. Reinhart and Horst [251] initially proposed that blunting of the catabolic pathway of  $1,25(\text{OH})_2\text{D}_3$  with high concentrations of



24,25(OH)<sub>2</sub>D<sub>3</sub> in rat cells would competitively inhibit further inactivation of 1,25(OH)<sub>2</sub>D<sub>3</sub>, resulting in an accumulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> and toxicity.

Clinical investigations of the downregulation of rat intestinal 24-OHase and its inhibition by calcitonin helped to elucidate a role of this hormone in potentiating the toxicity of vitamin D. 24-Hydroxylation is important in the inactivation of both 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> and, in the kidney, is largely regulated inversely by 1 $\alpha$ -hydroxylation [252]. In a study examining the effects of dietary calcium and vitamin D status on the regulation of intestinal 24-OHase enzyme and mRNA expression, rats were fed normal or low-calcium diets with variable amounts of vitamin D [253]. Half of the rats on the normal and low-calcium diets were administered pharmacological doses of vitamin D<sub>3</sub> (25,000 IU three times weekly). Excess vitamin D<sub>3</sub> resulted in significant elevations in plasma 25(OH)D<sub>3</sub> in both calcium groups, with a much larger increase noted in the normal calcium group. Hypercalcemia was most severe in the normal calcium and vitamin D<sub>3</sub> excess group, whereas rats in the low calcium and vitamin D<sub>3</sub> excess group had plasma calcium levels similar to the normal calcium group not treated with vitamin D. Among the rats treated with excess vitamin D<sub>3</sub>, because the normal calcium group was accompanied by an increased calcitonin concentration compared with the low calcium group, the authors suggested that the increased calcitonin in the normal calcium group may have suppressed 24-OHase activity, with resultant higher 25(OH)D<sub>3</sub> and calcium concentrations. This concept was further supported when rats, subjected to thyroparathyroidectomy, which eliminated endogenous calcitonin, were found to have higher concentrations of 24-OHase activity than the normal calcium and vitamin D<sub>3</sub> excess group. Through inhibition of intestinal 24-OHase activity, calcitonin could be associated with reduced turnover and catabolism of 25(OH)D<sub>3</sub>, thereby potentiating its toxicity. Thus, increased expression of 24-OHase activity in cases of pharmacological amounts of 25(OH)D<sub>3</sub> may be an important mechanism to counteract vitamin D toxicity. A key role for 24-OHase in preventing the development of vitamin D toxicosis was found in a relevant animal study. Growing dogs given 135-fold vitamin D<sub>3</sub> supplementation actually had a decrease in plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> levels by 40% as compared with controls, despite an increase in 1,25(OH)<sub>2</sub>D<sub>3</sub> production. This was attributed to an upgraded catabolism of 1,25(OH)<sub>2</sub>D<sub>3</sub> by 24-OHase, as evidenced by increased gene expression of renal and intestinal 24-OHase, thus providing an efficient hormonal counteraction [254]. Also relevant, St-Arnaud et al. [255] and Masuda et al. [256] showed that a targeted inactivating mutation of the 24-OHase gene in mice resulted in impaired catabolism of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Absence of the 24-OHase gene

during development also resulted in abnormal bone mineralization, with rescue of the phenotype by subsequent inactivation of the VDR. These experiments support a critical role of 24-OHase in preserving vitamin D homeostasis.

Furthermore, and as noted earlier, mutations in the *CYP24A1* gene encoding the 24-OHase enzyme in humans have been shown to be associated with idiopathic infantile hypercalcemia and in adults with hypercalcemia, hypercalciuria, nephrolithiasis, and elevated 1,25-dihydroxyvitamin D levels (see section “Decreased Clearance of 1,25-Dihydroxyvitamin D” under “Forms of Endogenous Vitamin D Toxicity”).

#### 4.5 Vitamin D-binding protein and the level of free metabolite in vitamin D toxicity

DBP is a specific transport protein that binds large quantities of the circulating vitamin D metabolites. Similar to the situation for other steroid hormones, fat-soluble compounds, and thyroid hormones, only a small fraction of the vitamin D metabolites circulate free in plasma. The binding affinity of the protein for the vitamin D metabolites is moderate, and the capacity is great (only 5% of binding sites on DBP are normally occupied). In addition, the various metabolites have different binding affinities for the protein, in the following sequence: 25(OH)D > 24,25(OH)<sub>2</sub>D > 1,25(OH)<sub>2</sub>D<sub>3</sub> [257]. Of note is the fact that the potent metabolite 1,25(OH)<sub>2</sub>D<sub>3</sub> has the least affinity for DBP, but the highest affinity for the intracellular VDR that triggers subsequent transcriptional events. Therefore, freeing bound 1,25(OH)<sub>2</sub>D<sub>3</sub> metabolite from DBP could promote its entry into various tissues and promote biological activity [258]. In states of vitamin D toxicity, the presence of elevated free 1,25(OH)<sub>2</sub>D<sub>3</sub> levels despite normal total 1,25(OH)<sub>2</sub>D<sub>3</sub> levels suggests that 1,25(OH)<sub>2</sub>D<sub>3</sub> is displaced from DBP by 25(OH)D, resulting in a rise of serum free calcitriol [259].

Evidence indicates that the biologically active form of the vitamin D steroid hormone is the free hormone that is accessible to cells [260]. Because of technical difficulties in measuring the free hormone, the determination of vitamin D status involves a measurement combining free vitamin D and DBP concentrations. In normal individuals, 85% of the total 1,25(OH)<sub>2</sub>D<sub>3</sub> is bound to DBP, 15% is bound to albumin, and 0.4% is free [261]. However, under conditions of altered or reduced albumin and DBP concentrations, as in liver or kidney disease, the free hormone may provide different information compared with the total measured concentration of vitamin D. Theoretically, total hormone concentration in such settings may erroneously suggest



deficiency of vitamin D with needless institution of replacement therapy. Bikle and colleagues noted that subjects with liver disease have reduced DBP concentrations with low total  $1,25(\text{OH})_2\text{D}_3$  and  $25(\text{OH})\text{D}$  levels, whereas free forms are normal [262,263].

Similarly, in certain forms of renal disease, the concentrations of DBP and vitamin D metabolites are reduced; thus, measurements of total hormone may provide an inaccurate reflection of vitamin D status. Koenig et al. [264] investigated free and total  $1,25(\text{OH})_2\text{D}_3$  concentrations in subjects with renal disease. Patients with nephrotic syndrome, with a range of degrees of renal failure including those on hemodialysis and peritoneal dialysis, were examined. The serum concentrations of total and free  $1,25(\text{OH})_2\text{D}_3$  correlated well with one another in patients with renal failure and those on hemodialysis. The concentrations of DBP and  $25(\text{OH})\text{D}$ , thus, were unaffected by renal function. The concentrations of total  $1,25(\text{OH})_2\text{D}_3$  accurately reflected free  $1,25(\text{OH})_2\text{D}_3$  in patients with varying degrees of renal failure when DBP levels remained normal. However, this did not hold true for the subjects with nephrotic syndrome or those on chronic peritoneal dialysis, who have losses of DBP and bound vitamin D metabolites into the urine or peritoneal fluid, respectively, with a rise in the percentage free  $1,25(\text{OH})_2\text{D}_3$  (also low-density-lipoprotein-related protein 2 or megalin may play a role).

Among subjects randomized to 20,000 IU vitamin  $\text{D}_3$  per week or placebo for 1 year, both serum total  $25(\text{OH})\text{D}$  and DBP were significantly lower among subjects with the DBP phenotype Gc2/Gc2 compared with other phenotypes, all of which included a Gc1S or Gc1F allele [265]. These levels were also lower in males than in females. Calculated or directly measured free  $25(\text{OH})\text{D}$  levels, while still lower among individuals with the Gc2/Gc2 phenotype, were not statistically significant from the other phenotypes. It should be noted that all participants in this study had vitamin D levels well within the normal range so the clinical significance of these differences is not clear. However, there may be situations such as cirrhosis or nephrotic syndrome where measurement of free metabolites may be important to avoid vitamin D toxicity when supplementation is instituted. Thus, in this context, the binding proteins of the vitamin D metabolites not only serve a transport function but also may provide a buffering mechanism to protect against toxicity [266].

## 5. Clinical manifestations of vitamin D toxicity

The clinical manifestations of vitamin D toxicity result primarily from hypercalcemia and reflect the

essential role of calcium in many tissues and targets, including bone, the cardiovascular system, nerves, and cellular enzymes. Initial signs and symptoms of hypervitaminosis D may be similar to other hypercalcemic states and include generalized weakness and fatigue. Central nervous system features may include confusion, difficulty in concentration, drowsiness, apathy, and coma [267]. Neuropsychiatric symptoms include depression and psychosis, which resolve following improvement of the hypercalcemia.

Hypercalcemia can affect the gastrointestinal tract and cause anorexia, nausea, vomiting, abdominal pain, and constipation. It can also induce hypergastrinemia. There is no evidence that peptic ulcers are more common than in any other form of hypercalcemia. Rarely, pancreatitis may be a presentation of either acute or chronic hypercalcemia.

In the heart, hypercalcemia may shorten the repolarization phase of conduction reducing the Q–T interval on the electrocardiogram (EKG). EKG changes in vitamin D toxicity have been mistaken for myocardial ischemia [268]. A more accurate EKG indication of the level of hypercalcemia is the Q–T interval corrected for rate. Bradyarrhythmias and first-degree heart block have been described, but are rare. Hypercalcemia may potentiate the action of digitalis on the heart [269].

Renal function is affected because high concentrations of calcium alter the action of vasopressin on the renal tubules. The net result is reduced urinary concentrating ability and a form of nephrogenic diabetes insipidus. This usually presents as polyuria, but rarely is the volume as high as that associated with central diabetes insipidus. Symptoms may include polydipsia, which is an expected consequence of polyuria. Hypercalciuria is one of the earliest signs of vitamin D toxicity and precedes the occurrence of hypercalcemia. The initial hypercalciuria may be ameliorated as renal failure progresses because of reduced calcium clearance. The pathophysiology of hypercalcemia can be rapidly worsened when volume depletion develops. When reduced renal blood flow occurs, less calcium is presented to the renal glomerulus, less calcium is filtered and excreted, and hypercalcemia can rapidly progress. Renal impairment from the hypercalcemia is reversible if it is of short duration. Chronic, uncontrolled hypercalcemia can lead to deposition of calcium phosphate salts in the kidney and permanent damage with eventual nephrocalcinosis. In an investigation of vitamin D–induced nephrocalcinosis, Scarpelli and colleagues [270] noted that cell damage, specifically in mitochondria, preceded intracellular calcium deposition. The hypercalcemia induced in rats by excessive vitamin D administration caused mitochondrial swelling, cell injury, and subsequent calcification.

Ectopic soft-tissue calcification can be a particular problem in hypervitaminosis D. The tendency toward soft-tissue calcification is compounded by the combination of hypercalcemia and hyperphosphatemia, often exceeding the solubility product of the two ions [271–273]. In rats exposed to excessive vitamin D, Hass and colleagues demonstrated that the pathological processes of vitamin D toxicity were related to dosage, length of time between doses, and duration of exposure [274]. For rats subjected to sublethal doses, generalized calcinosis was seen after only 8 days, when a total of 300,000 units of ergosterol was administered. Pathologically, bones appeared more brittle than normal, with increased cortical bone resorption, increased numbers of osteoclasts, and reduced numbers of osteoblasts. Abnormal calcium deposits were noted in the aorta and its major branches, heart, kidney, muscle, and respiratory tract. The earliest evidence of hypervitaminosis D was in the proximal aorta. Muscle tissue was the least resistant to calcification, with the order of decreasing susceptibility being smooth muscle > cardiac muscle > skeletal muscle [275]. The liver, brain, and pituitary were not affected by high doses of vitamin D. Permanent dental changes have also been reported with hypervitaminosis D, including enamel hypoplasia and focal pulp calcification [276]. Bone mineral density can be decreased due to excessive bone resorption [272,277], changes that can be reversed when vitamin D levels return to normal [278].

### 5.1 Diagnosis of vitamin D toxicity

With modern assays for calciotropic hormones, PTH, 25(OH)D, and 1,25(OH)<sub>2</sub>D<sub>3</sub>, one can readily differentiate vitamin D metabolite-mediated hypercalcemia from other causes of hypercalcemia. The circulating intact PTH level, measured by the two-site immunoradiometric assay (IRMA) or immunochemiluminometric assay (ICMA), should be suppressed in virtually all hypercalcemic disorders with the exception of primary hyperparathyroidism, familial hypocalciuric hypercalcemia, administration of lithium or thiazides, and renal failure. Although patients with malignancy-associated hypercalcemia tend to have a higher serum calcium concentration than those with other causes of hypercalcemia, diminished glomerular filtration rate and subsequent reduction in renal calcium excretion can dramatically increase the serum calcium level in any hypercalcemic patient. In contrast to the low serum phosphorus level in patients with hypercalcemia due to PTH or PTHrP, the serum phosphorus level is at the upper limit of normal or frankly elevated in patients with vitamin D metabolite-mediated hypercalcemia. This is due to increased intestinal absorption and reduced renal clearance of phosphate. An elevated 25(OH)D concentration with normal

1,25(OH)<sub>2</sub>D<sub>3</sub> level is indicative of toxicity with exogenously administered vitamin D or 25(OH)D. The serum 1,25(OH)<sub>2</sub>D level may be normally increased in patients with primary hyperparathyroidism due to the induction of renal 1 $\alpha$ -OHase by PTH. Abnormally high 1,25(OH)<sub>2</sub>D<sub>3</sub> levels, in the setting of suppressed PTH and hypercalcemia, indicate dysregulated production of 1,25(OH)<sub>2</sub>D<sub>3</sub> due to either granulomatous diseases, lymphoma, or toxicity with exogenous 1,25(OH)<sub>2</sub>D<sub>3</sub> or 1 $\alpha$ -OHD. In cases of hypercalcemia due to PTHrP or local osteolytic factors, the serum 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration may be suppressed, but the determinant here is not so much the PTHrP, which stimulates the 1 $\alpha$ -OHase but rather the extent of the hypercalcemia that suppresses the 1 $\alpha$ -OHase. In patients with hypercalcemia due to toxicity with other vitamin D analogs such as dihydrotachysterol (DHT) [279] and calcipotriol, the active metabolites may not be recognized by the antibodies used in the conventional assays for 1,25(OH)<sub>2</sub>D<sub>3</sub>.

The diagnosis of vitamin D toxicity can be made on clinical grounds. Detailed clinical and drug history are of paramount importance to make an early diagnosis. Most patients who are suffering from vitamin D toxicity are taking vitamin D for osteoporosis, hypoparathyroidism, pseudohypoparathyroidism, hypophosphatemia, osteomalacia, or renal osteodystrophy in excessive dosages or at too frequent dosing intervals. With the recent idea that vitamin D is protective of many diseases, vitamin D therapy is becoming very widespread in otherwise normal subjects. Therefore, one should have a high index of suspicion in patients who are being treated with pharmacological dosages of vitamin D or its metabolites. History should be obtained to rule out use of over-the-counter supplements such as Soladek, which may contain suprapharmacologic amounts of vitamin D at manufacturer-recommended dosages.

Patients with granulomatous diseases or lymphoma usually have widespread active disease when hypercalcemia develops. In such cases, the diagnosis is obvious at the time of presentation. However, exceptions do exist. In patients with unexplained hypercalcemia, if the 1,25(OH)<sub>2</sub>D<sub>3</sub> level is elevated and other more easily identifiable causes for this elevation such as primary hyperparathyroidism, pregnancy, and exogenous toxicity (by history) are excluded, measurement of angiotensin-converting enzyme level and a systemic search for lymph node enlargement, pulmonary, renal, hepatosplenic, ocular, central nervous system, and bone marrow granulomas or lymphoma should be made.

### 5.2 Treatment of vitamin D toxicity

Dietary calcium and vitamin D restriction and avoidance of exposure to sunlight and other ultraviolet light

sources should be advised to patients at high risk to develop vitamin D metabolite-mediated hypercalcemia. Those at risk include patients with granulomatous diseases and lymphoma whose disease is widespread and active and patients who are already hypercalciuric. Fluid intake should be encouraged. Daily dietary calcium intake should be minimized to approximately 400 mg or less in these patients. Any use of vitamin D supplements should be discontinued. The patient should be encouraged to use sunscreen (sun protection factor [SPF] > 15) as much as possible when outdoors. The calcium level should be monitored closely in patients who have a previous history of hypercalcemia or hypercalciuria, or who have recently taken diets enriched in vitamin D and calcium, or who have a recent history of excessive sunlight exposure. A reduction in oxalate intake may also be advisable, so as to prevent an increase in oxalate absorption and hyperoxaluria, which may increase the risk of kidney stone formation, despite a reduction in urinary calcium excretion [280]. Patients with isolated hypercalciuria without other indications for corticosteroid therapy can be considered for a therapeutic trial of a thiazide diuretic. Thiazide diuretics inhibit calcium excretion from the distal convoluted tubule and have been shown to decrease the risk of

recurrent nephrolithiasis in idiopathic hypercalciuria [281,282]. However, thiazide can increase circulating levels of calcium, by this mechanism.

When hypercalcemia develops, the aforementioned preventive measures will help to ameliorate the severity of hypercalcemia. Treatment measures are summarized in Table 81.2. Treatment of the underlying disease process is essential. General measures in those who are symptomatic include hydration with normal saline followed by the judicious use of a loop diuretic, like furosemide, in certain cases. As hypercalcemia induces volume depletion, it should be ensured that the patient is volume replete, even nearing volume overload, prior to administering furosemide. Specific inhibitors of bone resorption, such as bisphosphonates [272,277] and calcitonin, can be helpful.

Glucocorticoids have proved to be particularly effective in vitamin D intoxication, granulomatous diseases, and lymphoma. The precise mechanism of action of glucocorticoids in calcium homeostasis is not known. Nonetheless, they are useful because they (1) directly inhibit gastrointestinal absorption of calcium by decreasing the synthesis of calcium-binding protein (calbindin-D) and may downregulate intestinal VDR [283] and decrease active transcellular transport [284],

**TABLE 81.2** Treatment of vitamin D toxicity.

Intervention	Mechanism of action	Onset of action	Duration of action
<i>First-line treatments</i>			
Treatment of the underlying disease process	Variable	Variable	Variable
Isotonic intravenous saline	Restoration of intravascular volume Increases urinary calcium excretion	Within hours	During infusion
Loop diuretics	Increase urinary calcium excretion via inhibition of calcium reabsorption in the loop of henle	Within hours	During therapy
Glucocorticoids	Decrease intestinal calcium absorption Decrease 1,25-dihydroxyvitamin D production by activated mononuclear cells May alter hepatic vitamin D metabolism to favor the production of inactive vitamin D metabolites In some cases, direct anti-tumor effect (e.g., lymphomas, multiple myeloma)	2–5 days	Days to weeks
<i>Second-line treatments</i>			
Bisphosphonates	Inhibit bone resorption via interference with osteoclast function	24–72 h	2–4 weeks
Ketoconazole, fluconazole	Decrease 1,25-dihydroxyvitamin D production by activated mononuclear cells through inhibition of cytochrome P450	2–4 days	During therapy
Aminoquinolones (chloroquine and hydroxychloroquine)	Decrease 1,25-dihydroxyvitamin D production by activated mononuclear cells through unknown mechanism in granulomatous disease (shown to be ineffective in lymphoma)	Days to weeks	During therapy

Adapted from Ref. [298].

(2) increase urinary excretion of calcium [285], and (3) may alter hepatic vitamin D metabolism to favor the production of inactive vitamin D metabolites, resulting in lower concentrations of 25(OH)D [286]. Evidence also suggests that they may increase the degradation of 1,25(OH)<sub>2</sub>D<sub>3</sub> at the receptor sites [287]. Glucocorticoids may also limit osteoclastic bone resorption [288]. Institution of glucocorticoid therapy results in prompt decline of the circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations within 3–4 days [101]. Patients with nonhematological malignancies and those with primary hyperparathyroidism do not usually respond to glucocorticoids.

Aminoquinolones (chloroquine and hydroxychloroquine) are also capable of reducing the 1,25(OH)<sub>2</sub>D<sub>3</sub> and calcium concentrations in patients with sarcoidosis [289–292]. The theoretical advantage of aminoquinolones over glucocorticoids is that correction of the 1,25(OH)<sub>2</sub>D<sub>3</sub> should result in recovery of at least some of the bone density lost to the disease without the countervailing adverse skeletal effects of glucocorticoids [278]. In lymphoma cells, however, aminoquinolones do not have the same regulatory effects on the excess 1,25(OH)<sub>2</sub>D<sub>3</sub> as they do in granulomatous disease. In the presence of lymphoma, it is preferable to use glucocorticoid-containing antitumor regimens [293]. Owing to the limited experience with aminoquinolone drugs as antihypercalcemic agents and their potential side effects, they should be reserved for patients in whom steroid therapy is unsuccessful or specifically contraindicated. Ketoconazole, an antifungal agent, in high dosages can inhibit the mitochondrial cytochrome P450-linked 25(OH)D 1 $\alpha$ -OHase irrespective of whether it is renal [279] or extrarenal as in sarcoidosis [294] and tuberculosis [141]. Case reports of ketoconazole use in conjunction with glucocorticoids in sarcoidosis suggest that the addition of ketoconazole may reduce steroid requirements [295,296]. Ketoconazole has been used successfully to decrease the hypercalcemia, hypercalciuria, and elevated serum 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in patients with CYP24A1 mutations [229,297]. Use of ketoconazole is often limited by hepatic side effects and adrenal insufficiency unless used concomitantly with glucocorticoids. Fluconazole has also been described for treatment and may be effective at low doses [232].

## 6. Conclusions

Vitamin D toxicity is not a common cause of hypercalcemia, but it can be life-threatening if not identified promptly. When the two major etiologies of hypercalcemia, primary hyperparathyroidism and malignancy, are excluded, vitamin D toxicity becomes an important diagnostic consideration. There are many forms of exogenous

and endogenous vitamin D toxicity. Inadvertent excessive use of pharmaceutical preparations is the most common cause of exogenous toxicity. Excessive amounts of the parent compound, vitamin D, can be most difficult to manage as compared with toxicity due to the metabolites 25(OH)D or 1,25(OH)<sub>2</sub>D<sub>3</sub>. Extensive lipid solubility of vitamin D accounts for its long half-life and tendency for prolonged hypercalcemia. New clinical applications of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its synthetic analogs have been accompanied by the increased potential for toxicity. Endogenous etiologies may result from ectopic production of 1,25(OH)<sub>2</sub>D<sub>3</sub> in multiple diseases including sarcoidosis, tuberculosis, rarer granulomatous conditions, inflammatory reactions to foreign bodies, or in lymphoma. Genetic testing has discovered mutations in the CYP24A1 gene responsible for decreased clearance of 1,25(OH)<sub>2</sub>D<sub>3</sub>, which can lead to hypercalcemia. Many different mechanisms have been proposed to account for vitamin D toxicity, including the vitamin D metabolite itself, VDR number, activity of 1 $\alpha$ -OHase, inhibition of vitamin D metabolism, and the capacity of DBP to bind vitamin D metabolites. Mounting evidence that higher levels of vitamin D may have beneficial effects on bone and cellular health may predispose to enhanced administration of vitamin D in the future and thereby increased frequency of vitamin D toxicity.

## 7. Summary points

- Exogenous vitamin D toxicity is a result of excessive intake of parent vitamin D, its metabolites, or vitamin D analogs.
- Endogenous vitamin D toxicity may result from ectopic production of 1,25(OH)<sub>2</sub>D<sub>3</sub> in multiple diseases or from decreased clearance of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the setting of CYP24A1 gene mutations.
- Different proposed mechanisms of vitamin D toxicity include metabolites of vitamin D, vitamin D receptor concentrations, activity of 1 $\alpha$ -OHase, inhibition of vitamin D metabolism, and the capacity of vitamin D-binding protein.
- The clinical manifestations of vitamin D toxicity result primarily from hypercalcemia, and the diagnosis can be made based on the clinical history and measurement of PTH, 25(OH)D, and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels.
- Initial treatment of hypercalcemia due to vitamin D toxicity includes restricting dietary intake of vitamin D and calcium, withholding supplementations, and hydration with isotonic saline. Depending on the exact mechanism, loop diuretics, bisphosphonates, calcitonin, glucocorticoids, aminoquinolones, and ketoconazole may also be added for management.



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## Further reading

*The reference list is extensive and includes essentially all information related to the topic, to date. The reader might want to refer to other information about vitamin D that while not related to hypercalcaemia directly, may nevertheless, enhance the reader's appreciation of the topic.*

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# Vitamin D and Paget's disease

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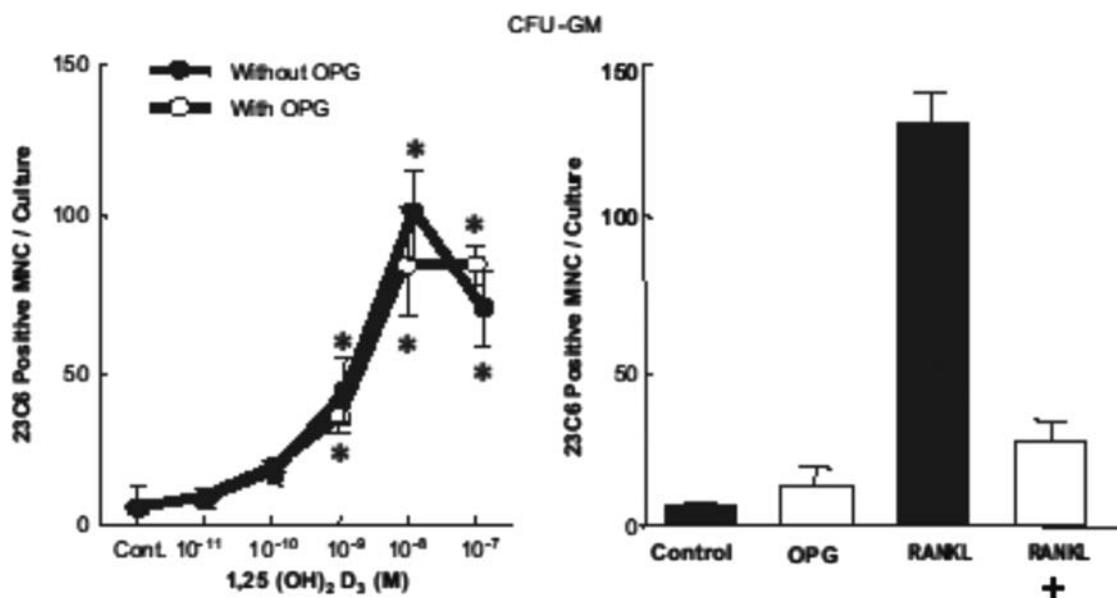
## OBJECTIVES

- Expression of TAF 12 in osteoclast precursors from Paget's disease (PD) patients contributes to their hyperresponsivity to low concentrations of  $1,25(\text{OH})_2\text{D}_3$
- Understanding the mechanisms responsible for the increased vitamin D responsivity of OCL precursors from patients with PD, and the effects of VDR-mediated transcription on OCL activity in PD.
- Understand the importance of correcting low serum concentrations of vitamin D and the occurrence of acute phase reactions (APR) to bisphosphonates in PD patients

## 1. Introduction

Paget's disease (PD) is a common bone disorder that was originally described by Sir James Paget over 100 years ago [1]. PD is characterized by highly localized areas of bone resorption coupled with exuberant new bone formation. Bone lesions in PD contain markedly increased numbers of abnormal osteoclasts (OCLs) and large numbers of osteoblasts that rapidly form new bone in response to the increased bone resorption. The bone that is formed in PD is highly abnormal woven bone and is of poor quality because of the rapid bone formation. However, the OCL is the primary cell involved in the pathogenesis of PD. OCLs in PD lesions are increased in number and size [2] and contain up to

100 nuclei per OCL. In contrast, normal human OCL contains between 3 and 10 nuclei per osteoclast. Pagetic OCLs also contain paramyxoviral inclusions [3] that are not present in other bone marrow cells or in nonpagetic bones of these patients. Further, several studies have shown that these nuclear inclusions cross-react with antibodies to the respiratory syncytial virus and/or measles virus nucleocapsid proteins. Abe and coworkers also found the budding-off of viral particles from pagetic OCLs [4]. These results suggested a possible viral etiology for PD. We previously showed that pagetic OCLs from 70% of PD patients we tested contain measles virus (MV) transcripts [5], and demonstrated that OCL formed by normal human OCL precursors transfected with the measles virus nucleocapsid protein gene (MVNP) have many of the features of pagetic OCL, including responsivity to  $1,25(\text{OH})_2\text{D}_3$  and rank ligand (RANKL), production of high levels of IL-6 and TAF12, a vitamin D nuclear receptor (VDR) coactivator [6]. However, there is a strong genetic component also involved [7] with about 30% of patients having an affected first-degree relative, and large kindreds of Paget's patients have been reported. Interestingly, OCL formed in marrow cultures from PD patients harboring mutations linked to PD but not expressing MVNP, are hyperresponsive to RANKL but not to  $1,25(\text{OH})_2\text{D}_3$  [8], and do not express high levels of TAF12 [8]. However, marrow stromal cells from PD patients harboring the  $\text{p62}^{\text{P392L}}$  mutations express high levels of TAF12 and produce increased amounts of RANKL in response to  $1,25(\text{OH})_2\text{D}_3$ . In this chapter, we will focus on the mechanisms responsible for the increased vitamin D responsivity of OCL precursors from patients with PD and the effects of VDR-mediated transcription on OCL activity in PD.



**FIGURE 82.1** Osteoprotegerin (OPG) does not inhibit osteoclast (OCL) formation in cultures of purified human OCL precursors. Highly purified OCL precursors (CFU-GM) that do not contain stromal cells from normal donors ( $10^6$  cells/well) were cultured with  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-11}$ – $10^{-7}$  M) or rank ligand (RANKL) (50 ng/mL) in the presence or absence of OPG (50 ng/mL). After 2 weeks, the cells were fixed and stained with the 23C6 monoclonal antibody, which identifies OCLs. The results are expressed as mean  $\pm$  SD for quadruplicate cultures. \*,  $P < .05$  compared to control lacking added  $1,25(\text{OH})_2\text{D}_3$ . CFU-GM, granulocyte–macrophage colony-forming unit; SD, standard deviation

## 2. Mechanism of action of $1,25(\text{OH})_2\text{D}_3$ on osteoclast formation

Vitamin D plays a major role in bone homeostasis.  $1,25(\text{OH})_2\text{D}_3$ , the active form of vitamin D<sub>3</sub>, binds the classical VDR in the cytoplasm at high affinity, and the complex then translocates to the nucleus.  $1,25(\text{OH})_2\text{D}_3$  promotes heterodimerization of VDR with the retinoid receptor, which recruits coactivators and releases corepressors from vitamin D response element (VDRE) present in the promoters of vitamin D target genes. The VDR-associated coactivators then recruit the transcription factor TFIID multiprotein complex, composed of TATA-binding protein (TBP) and a series of TBP-associated factors (TAFs), which in turn recruit the RNA Pol II transcription complex to the promoter. Formation of this complex results in transcription of vitamin D-responsive genes [9–12].

$1,25(\text{OH})_2\text{D}_3$  has several important effects on bone remodeling. Among these, it enhances calcium (Ca) absorption through the gut, and it increases RANKL production and suppresses osteoprotegerin (OPG) expression by marrow stromal cells and osteoblasts. We showed that OCL precursors express VDR [13] and found that  $1,25(\text{OH})_2\text{D}_3$  may directly stimulate human OCL precursors to form OCLs. This conclusion is based on our findings that OCL formation by unfractionated normal human bone marrow mononuclear cells treated with  $1,25(\text{OH})_2\text{D}_3$  can only be partially inhibited by OPG. In contrast, when these marrow cultures are

treated with RANKL rather than  $1,25(\text{OH})_2\text{D}_3$ , OPG totally inhibits OCL formation. Further, OPG does not affect OCL formation by highly purified normal human OCL precursors that do not contain stromal cells, treated with  $1,25(\text{OH})_2\text{D}_3$ . In contrast, OPG totally blocks OCL formation when these highly purified human OCL precursors are treated with RANKL. These results demonstrate that  $1,25(\text{OH})_2\text{D}_3$  stimulates human OCL formation in human marrow cultures both directly and indirectly through its capacity to induce RANKL (Fig. 82.1).

## 3. Increased levels of TAF12 in osteoclast precursors from Paget's disease patients contribute to their hyperresponsivity to $1,25(\text{OH})_2\text{D}_3$

One of the earliest findings in our studies of OCL formation in PD was that OCL precursors from Paget's patients were hyperresponsive to  $1,25(\text{OH})_2\text{D}_3$  and formed OCL-like cells in marrow culture at concentrations of  $1,25(\text{OH})_2\text{D}_3$  that were at least 1–2 logs less than those required for OCL formation in normal marrow cultures [13]. We found, using highly purified populations of early OCL precursors from Paget's patients, that the high responsivity of OCL precursors to  $1,25(\text{OH})_2\text{D}_3$  appeared to be an intrinsic property of early OCL precursors, the granulocyte–macrophage colony-forming unit (CFU-GM)–derived cells [14].

However, the mechanism responsible for the enhanced  $1,25(\text{OH})_2\text{D}_3$  responsiveness of these early precursors was unknown. We showed that the enhanced sensitivity of pagetic OCL precursors was not because of increased VDR numbers [15], and thought it was unlikely that Paget's patients have a mutated VDR that has an intrinsically increased affinity for  $1,25(\text{OH})_2\text{D}_3$ . This conclusion was based on multiple reports that although mutations that predispose to PD have been identified on more than six different chromosomal loci, none of these coincide with the chromosomal location of VDR. Further, the majority of PD patients who do not have a genetic predisposition to PD appear to have OCL precursors that are hyperresponsive to  $1,25(\text{OH})_2\text{D}_3$ .

To investigate the mechanisms responsible for the enhanced  $1,25(\text{OH})_2\text{D}_3$  responsiveness of PD OCL precursors, we incubated a GST (glutathione s-transferase)–VDR chimeric protein with lysates from marrow cells of involved bone from PD patients [16,17] and isolated TAF12 (formerly termed TAF<sub>II</sub>-17). Transduction of TAF12 into NIH3T3 cells, specifically increased VDR transcriptional activity. Furthermore, mammalian two-hybrid studies showed that TAF12 bound VDR and enhanced VDR-mediated transcription [18], demonstrating that TAF12 acted like a novel VDR coactivator.

TAF12 is a member of the TFIID transcription complex that was thought to be only responsible for promoter recognition and directing RNA Pol II to core promoters in response to activators of transcription [19,20]. This function of TFIID was considered universal rather than gene-specific because TFIID is expressed in all tissues. However, several studies suggested that different combinations of TAFs are mobilized in response to different growth stimuli and that some TAFs (e.g., TAF4, TAF7, TAF10, and TAF11) can act as specific coactivators of genes [21]. The histone fold domains of TAFs play a critical role in their physical association with gene promoters. These domains interact to form heterodimers between particular pairs of TAFs (e.g., TAF4 and TAF12), which associate to form histone octamer–like substructures that bind to core promoters. Interestingly, TFIID components can be cell–type-specific and regulate specific genes. For example, TAF4b (formerly TAF<sub>II</sub>-105 and a TAF4 paralogue) is highly expressed in the testes and ovary and regulates the transcription of specific genes in granulosa cells. Mice lacking TAF4b are sterile [22]. Other alternate forms of the TFIID transcription complex have also been shown to act as selective activators of cell–type-specific gene expression required for cell differentiation. For example, in *Drosophila* five tissue-specific TAF paralogues have been detected for TAFs 4, 5, 6, 8, and 12 [23]. TAF4 and TAF12 interact structurally and can regulate a testis-specific gene expression program in primary spermatocytes required for their terminal

differentiation [23]. Similarly, TAF3 and TRF3 can act as a novel core transcription complexes for the differentiation of myoblasts to myotubules [24]. These results support the notion that TAF12 may play an important role in tissue-specific gene expression involved in OCL differentiation.

### 3.1 Measles virus infection of osteoclast precursors induces TAF12

To determine if MV infection increased the responsiveness of OCL precursors to  $1,25(\text{OH})_2\text{D}_3$  and resulted in upregulation of TAF12, a cellular MV receptor, human CD46, was targeted to cells in the OCL lineage in transgenic mice using the mouse tartrate-resistance acid phosphatase (TRACP) gene promoter. Targeting hCD46 to murine cells allows MV infection. The MV-infected cells were hyperresponsive to  $1,25(\text{OH})_2\text{D}_3$  and expressed TAF12. OCLs formed by MV-infected marrow cells from TRACP–CD46 mice expressed several characteristics of pagetic OCLs [25]. These results suggest that MV infection of OCL precursors can induce TAF12.

### 3.2 Transgenic mice with tartrate-resistance acid phosphatase–driven measles virus nucleocapsid gene expression in OCLs also express TAF12

Because we found that OCLs from patients with PD expressed MVNP, we then determined if the persistent expression of the MVNP gene could induce changes in the bone similar to those found in PD. The MVNP gene was targeted to cells in the OCL lineage in transgenic mice using the TRACP promoter. These mice developed bone abnormalities similar to those seen in PD at 4–16 months of age (TRACP–MVNP mice) [26]. Significantly increased numbers of OCLs were formed in marrow cultures from TRACP–MVNP mice compared to marrow cultures from WT mice treated with  $1,25(\text{OH})_2\text{D}_3$ . Further, TRACP–MVNP marrow cultures formed OCLs at concentrations of  $1,25(\text{OH})_2\text{D}_3$  that were significantly lower than those required for WT marrow cultures, with OCL formation occurring in MVNP cultures at  $10^{-11}$ – $10^{-12}$  M  $1,25(\text{OH})_2\text{D}_3$ . In addition, the OCL precursors from TRACP–MVNP, but not WT mice, expressed high levels of TAF12 mRNA. Finally, the number of nuclei per OCL was also significantly increased in marrow cultures from TRACP–MVNP mice compared to WT mice, and the OCLs that formed were larger than those formed in WT marrow cultures.



### 3.3 Transduction of an antisense measles virus nucleocapsid protein construct into osteoclast precursors from patients with Paget's disease also reduced expression of TAF12

To examine if TAF12 expression was required for the  $1,25(\text{OH})_2\text{D}_3$  hyperresponsivity of PD OCL precursors, we determined the effects of transducing an antisense MVNP construct (AS-MVNP) into OCL precursors from patients with PD on OCL formation. Transfection of MVNP-expressing OCL precursors with an AS-MVNP construct decreased expression of MVNP mRNA by nearly 70% and resulted in an 80% reduction in TAF12 expression, levels as well as significant reductions in OCL formation, nuclei/OCL, and IL-6 production induced by  $1,25(\text{OH})_2\text{D}_3$ , as compared to cells transfected with the scrambled control antisense construct (AS-CONT) [27]. The OCLs that formed by the AS-MVNP transduced PD patient-derived OCLs were not hypernucleated. AS-MVNP also inhibited the increased bone resorption by OCLs formed in marrow cultures from MVNP-expressing PD patients when compared with normal OCLs. AS-MVNP had no effect on OCL formation, OCL morphology, bone resorption, or IL-6 production by PD patients with a mutation in p62, linked to PD, but not expressing MVNP or normal OCL precursors [27].

### 3.4 Effects of IL-6 on TAF12 expression by osteoclast precursors

IL-6 levels are markedly increased in OCL precursors from patients with PD [28] and MVNP-transduced normal OCL precursors. IL-6 is also a major mediator of human OCL formation, and high levels of IL-6 can induce hypermultinucleated OCLs that display many of the features of pagetic OCLs [29] and can induce TAF12 mRNA in normal OCL precursors. Further, treatment of pagetic OCL precursors with an antibody to IL-6 decreases OCL formation [30].

To further investigate the role of IL-6 in TAF12 expression by OCL precursors, we targeted the overexpression of IL-6 to the OCL lineage in transgenic mice using the TRACP promoter. TRACP-IL-6 mice expressed twofold higher levels of IL-6 [31], and the OCL precursors were hyperresponsive to  $1,25(\text{OH})_2\text{D}_3$ . Measurement of TAF12 expression in TRACP-IL-6 mice demonstrated that TAF12 levels were increased approximately twofold compared with wild-type (WT) littermates. To further assess the role of IL-6 in  $1,25(\text{OH})_2\text{D}_3$  responsivity of OCL precursors, TRACP-MVNP mice, which express a pagetic phenotype and high levels of IL-6, were bred to IL-6<sup>-/-</sup> mice [32]. In contrast to MVNP mice, OCL

precursors from MVNP/IL-6<sup>-/-</sup> mice were not hyperresponsive to  $1,25(\text{OH})_2\text{D}_3$ , expressed low levels of TAF12 and formed OCL that contained a similar number of nuclei/OCL as WT mice and did not express a pagetic phenotype. These results demonstrate that IL-6 induces many of the effects of MVNP on OCL formation.

Thus, loss of IL-6 expression in TRACP-MVNP mice decreased TAF12 expression, reversed the hyperresponsivity of OCL precursors to  $1,25(\text{OH})_2\text{D}_3$  and resulted in the loss of the pagetic phenotype in OCLs formed in marrow cultures from these mice [27]. These results demonstrate that IL-6 upregulates TAF12 in OCL precursors, and that loss of IL-6, even when MVNP is present in these OCL precursors, results in loss of TAF12 expression.

### 3.5 Overexpression of TAF12 is sufficient to induce $1,25(\text{OH})_2\text{D}_3$ hyperresponsivity in human osteoclast precursors

To further assess the role of TAF12 in OCL formation, we determined the effects of overexpression of TAF12 in normal human OCL precursors. These experiments allowed a direct determination of the capacity of increased levels of TAF12 to mediate  $1,25(\text{OH})_2\text{D}_3$  hypersensitivity in OCL precursors in vitro and to determine the contribution of TAF12 to the development of pagetic-like OCLs.

The cDNA for TAF12 was synthesized by RT-PCR from OCL precursor cells of PD patients and inserted into a retroviral construct [33]. The TAF12-expressing virus was transduced into normal human marrow cells. TAF12-transduced normal OCL precursors expressed twofold higher TAF12 mRNA levels compared with empty vector (EV)-transduced OCL precursors. In addition, an MVNP-retrovirus construct was transduced into human nonadherent mononuclear cells to compare TAF12 overexpressing OCL precursors with MVNP-expressing OCLs. The OCL precursors were treated with  $10^{-11}$ – $10^{-7}$   $1,25(\text{OH})_2\text{D}_3$ , and the number and characteristics of the OCLs formed and levels of CYP24A1 mRNA were determined and compared with EV-transduced OCLs. The TAF12-transduced cells formed increased numbers of OCLs that were hypersensitive to  $1,25(\text{OH})_2\text{D}_3$ , but in contrast to MVNP-expressing cells, did not contain increased numbers of nuclei per OCL or produce high levels of IL-6 ( $47 \pm 1$  pg/mL vs.  $269 \pm 11$  pg/mL, TAF12- vs. MVNP-transduced cells) [34]. Similarly, the EV-transduced cells did not produce detectable levels of IL-6. We then compared the bone-resorbing capacity of TAF12-transduced OCL precursors treated with  $1,25(\text{OH})_2\text{D}_3$  to MVNP-transduced OCL precursors. OCLs formed by MVNP-transduced OCL precursors treated with

$1,25(\text{OH})_2\text{D}_3$  had a markedly increased bone-resorbing capacity per OCL. In contrast, the bone resorption capacity per OCL formed by TAF12-transduced OCL precursors was similar to those from EV-transduced OCL precursors. These results strongly suggested that increased TAF12 expression is not sufficient to induce a pagetic phenotype in OCLs but is sufficient to induce hyperresponsivity to  $1,25(\text{OH})_2\text{D}_3$  in OCL precursors.

### 3.6 Osteoclast precursors from TRACP–TAF12 transgenic mice are hyperresponsive to $1,25(\text{OH})_2\text{D}_3$

To further characterize the contribution of TAF12 to OCL formation in PD, we generated TRACP–TAF12 transgenic mice in which TAF12 expression is targeted to the OCL lineage with the TRACP promoter. When bone marrow cells from TRACP–TAF12 and TRACP–MVNP mice were cultured with  $1,25(\text{OH})_2\text{D}_3$ , OCLs formed at low concentrations ( $10^{-10}$  M) of  $1,25(\text{OH})_2\text{D}_3$ , a concentration that does not induce OCL formation in marrow cultures from WT mice. OCLs formed from TRACP–MVNP marrow exhibited markedly elevated nuclear numbers per OCL in response to  $1,25(\text{OH})_2\text{D}_3$ , but the nuclear number per OCL in TRACP–TAF12 mice was similar to WT OCLs. To determine if these OCL precursors demonstrated enhanced VDR-mediated transcription at low concentrations of  $1,25(\text{OH})_2\text{D}_3$ , we measured the expression of *CYP24A1* in response to varying concentrations of  $1,25(\text{OH})_2\text{D}_3$ . OCL precursors from both TRACP–MVNP and TRACP–TAF12 mice showed increased *CYP24A1* expression compared with OCL precursors from WT mice when treated with low concentrations of  $1,25(\text{OH})_2\text{D}_3$ . IL-6 production by OCL precursors treated with  $1,25(\text{OH})_2\text{D}_3$  was also increased in both TRACP–MVNP and TRACP–TAF12 OCL precursors compared with WT mice but to a lesser extent in the TRACP–TAF12 derive cells. Histomorphometric analysis of bones from TRACP–TAF12 mice showed only mild increases in OCL formation and no morphologic changes in characteristics of PD [34].

### 3.7 Effects of transduction of a TAF12 antisense construct on OCL formation in marrow cultures from PD patients expressing MVNP in their OCL precursors

To determine the contribution of TAF12 to the hyper-sensitivity to  $1,25(\text{OH})_2\text{D}_3$  in OCL precursors from PD patients, we transduced a retrovirus construct containing an antisense to TAF12 into MVNP-expressing OCL precursors from PD patients and healthy controls

because our previous studies showed that almost all MVNP-expressing OCL precursors from PD patients were hyperresponsive to  $1,25(\text{OH})_2\text{D}_3$  and expressed increased levels of TAF-12. The TAF12 antisense construct decreased TAF12 expression by more than 80%, as well as  $1,25(\text{OH})_2\text{D}_3$  hypersensitivity of the PD OCL precursors, to levels that were like the effects of antisense MVNP.

When MVNP-expressing Paget's patient CFU-GM was cultured for 14 days in the presence or absence of TAF12 antisense construct or a control antisense construct (AS-CONT) and treated with  $10^{-11}$ – $10^{-7}$  M  $1,25(\text{OH})_2\text{D}_3$ , OCL formation was decreased by 37%–64% by antisense TAF12 (Fig. 82.2). Thus, transfection of an antisense TAF12 construct into OCL precursors from PD patients expressing MVNP decreased their hypersensitivity to  $1,25(\text{OH})_2\text{D}_3$  [34].

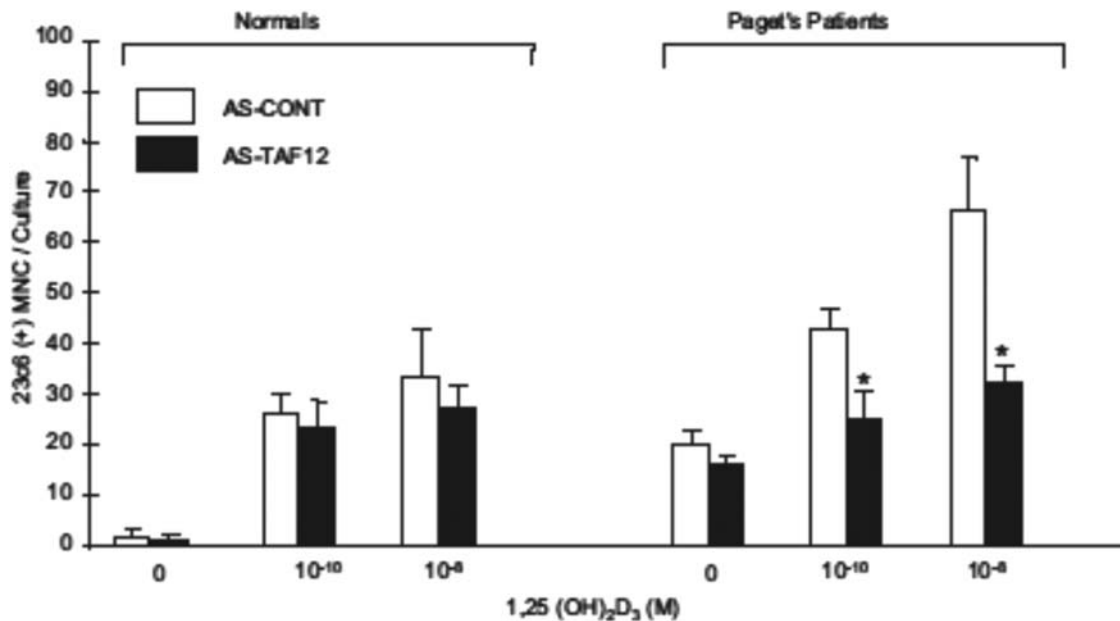
## 4. Mechanism of action of TAF12 in the increased $1,25(\text{OH})_2\text{D}_3$ responsivity of OCL precursors from Paget's patients

### 4.1 TAF12 binds the *Cyp24A1* promoter in osteoclast precursors

The increased expression of TAF12 induced by the high IL-6 levels in MVNP-expressing OCL is one mechanism underlying their hyperresponsivity to  $1,25(\text{OH})_2\text{D}_3$  in PD. To further characterize the mechanisms responsible for the effects of TAF12 on  $1,25(\text{OH})_2\text{D}_3$  responsivity, we determined if TAF12 binds the *CYP24A1* promoter. Chromatin immunoprecipitation studies were performed on the *CYP24A1* promoter in both TRACP–MVNP and WT OCL precursors, using an anti-TAF12 antibody and primers flanking the two VDREs.  $1,25(\text{OH})_2\text{D}_3$  induced TAF12 binding to the *CYP24A1* promoter in TRAP–MVNP as well as WT OCL precursors, but with both basal and induced levels of TAF12 binding were much higher in the OCL precursors from TRACP–MVNP mice [34].

### 4.2 TAF12 interacts with ATF7

Several studies suggested that TAF12 is a functional partner of ATF7 and TAF4. ATF7 binds as a homodimer to cAMP response element sequences (TGACGTCA) and can also heterodimerize with members of the Jun and Fos families to bind TPA response element TRE sequences (TGACTCAG). Hamard and colleagues [35] reported that overexpressed TAF12 directly interacts with ATF7 and potentiates ATF7-induced transcriptional activation of ATF7-driven genes. Further, coimmunoprecipitation studies supported the physical interaction of



**FIGURE 82.2** Osteoclast (OCL) formation induced by  $1,25(\text{OH})_2\text{D}_3$  is reduced in cultures of antisense TAF12 (AS-TAF12) transduced human OCL precursors from Paget's disease (PD) patients but not from normals. AS-TAF12 or scrambled antisense-transduced OCL precursors from three PD patients or two normals were cultured in methylcellulose with recombinant GM-CSF and G418. G418-resistant CFU-GM-derived  $\text{CD}11\text{b}^+$  cells were then cultured with varying concentrations of  $1,25(\text{OH})_2\text{D}_3$  for 3 weeks. The cells were then fixed and stained with the 23C6 monoclonal antibody, which identifies OCL. The results represent the mean  $\pm$  SD of aggregate data from three MVNP<sup>+</sup> PD patients and two normals. \*,  $P < .01$  compared to scrambled antisense transduced cells. CFU-GM, granulocyte-macrophage colony-forming unit; GM-CSF, granulocyte-macrophage colony-stimulating factor; MVNP, measles virus nucleocapsid protein; SD, standard deviation

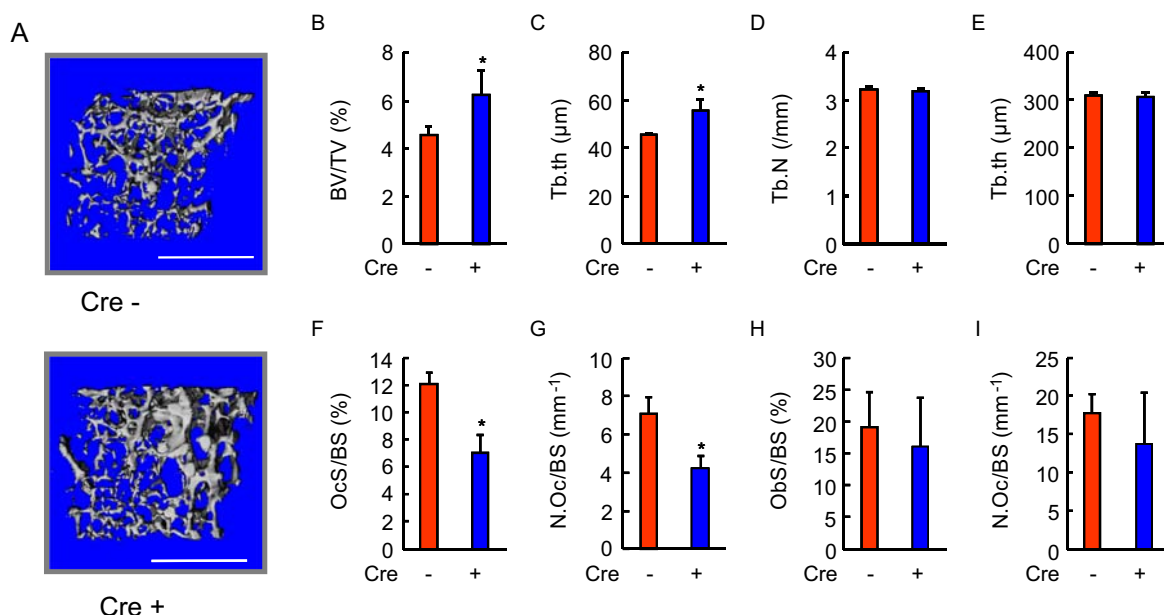
ATF7 with TAF12 on an ATF7-responsive promoter in the absence of overexpression of either protein. Importantly, TAF12-dependent ATF7-regulated transcriptional activation was competitively inhibited by TAF4, which binds both ATF7 and TAF12. ATF7, like TAF12, is also increased in OCL precursors from TRACP-MVNP mice and in MVNP-expressing NIH3T3 cells. High levels of ATF7 expression were detected in MVNP-expressing cell lysates and were not further increased by  $1,25(\text{OH})_2\text{D}_3$ . Expression of TAF4 was not affected by MVNP. Immunoprecipitation of lysates from either WT or TRACP-MVNP OCL precursors with an anti-TAF12 antibody followed by blotting with an anti-ATF7 antibody or vice versa revealed that TAF12 and ATF7 physically interact in OCL precursors. These results demonstrate that TAF12 and ATF7 physically interact in OCL precursors, and that the ratio of TAF12 to TAF4 is increased by MVNP. As noted above, a key VDR target gene, *CYP24A1*, is the first gene activated by VDR and deactivates  $1,25(\text{OH})_2\text{D}_3$  to control the transcriptional activity of VDR. We also found that knockdown of ATF7 decreased the sensitivity of *CYP24A1* to  $1,25(\text{OH})_2\text{D}_3$  as well as TAF12 levels in MVNP-expressing cells. Thus, the interaction of ATF7 with TAF12 may be involved in the upregulation of TAF12 and the hypersensitivity of OCL precursors to  $1,25(\text{OH})_2\text{D}_3$ .

### 4.3 TAF12 enhances vitamin D nuclear receptor content and half-life in cells

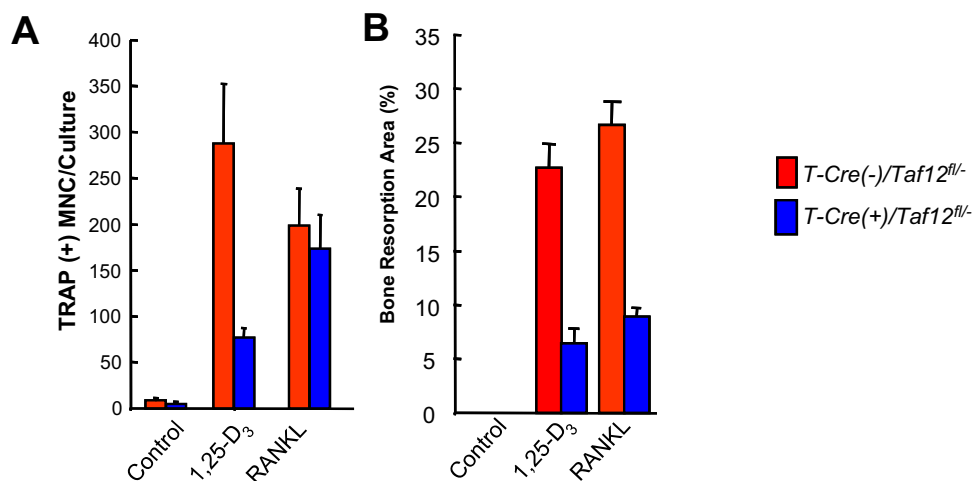
Because stabilization of the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex by TAF12 and/or ATF7 is a potential mechanism for increased VDR responsiveness in MVNP-expressing cells [36], we measured VDR content by Western blot in MVNP-transfected NIH3T3 cells (MVNP-NIH3T3) and empty vector-transfected cells (EV-NIH3T3). We found that  $1,25(\text{OH})_2\text{D}_3$  increased VDR content in both cell types. Further, physiological concentrations of  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-10}$  M) markedly enhanced the stability of VDR in MVNP-NIH3T3 cells that also expressed increased levels of TAF12. In contrast, high concentrations of  $1,25(\text{OH})_2\text{D}_3$  (over  $10^{-8}$  M) were required to enhance VDR stability in EV-NIH3T3 cells [34]. Moreover, hyperresponsivity of MVNP-NIH3T3 cells to  $1,25(\text{OH})_2\text{D}_3$  was diminished by transfection of TAF12 siRNA, which also decreased VDR content. Results obtained using bone marrow from TRACP-MVNP and TRACP-TAF12 mice demonstrated that when TAF12 expression was increased,  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-12}$ – $10^{-7}$  M) markedly increased VDR content (Fig. 82.3). These results suggest that stabilization of the  $1,25(\text{OH})_2\text{D}_3$ -VDR complex may contribute to the hyperresponsivity of OCL precursors to  $1,25(\text{OH})_2\text{D}_3$  that overexpress TAF12 [34].







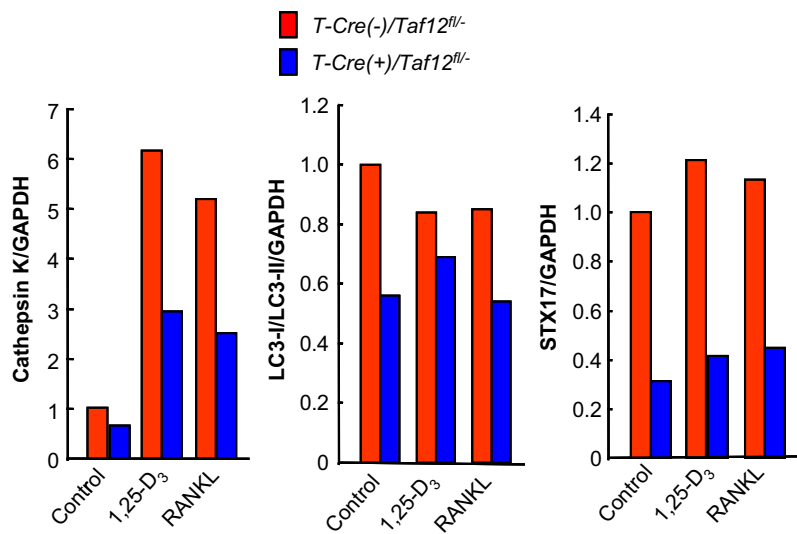
**FIGURE 82.4** *Trap-Cre(+)/Taf12<sup>fl/-</sup>* mice exhibit increased bone mass, and decreased bone resorption. (A) Representative micro-CT images of distal femur from mice at 12 months of age. Scale bar, 1 mm. (B–I) bone morphometric analysis of 8-month-old *Trap-Cre(-)/Taf12<sup>fl/-</sup>* (*n* = 3) and *Trap-Cre(+)/Taf12<sup>fl/-</sup>* (*Taf12cKO*) (*n* = 4) mice. BV/TV, bone volume per total volume; N.Oc/BS, osteoclast number per bone surface; N.Oc/BS, osteoclast number per bone surface; ObS/BS, osteoblast surface per bone surface; OcS/BS, osteoclast surface per bone surface; Tb.th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation. Data represent the mean value of the indicated parameter ± SD. All parameters were compared to *Trap-Cre(-)/Taf12<sup>fl/-</sup>* mice by Mann-Whitney U-test (\**P* < .05).



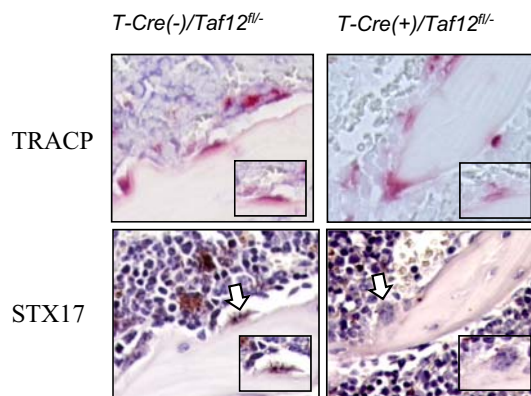
**FIGURE 82.5** Osteoclast formation and bone resorption by *Trap-Cre/Taf12<sup>fl/-</sup>* OCLs. (A) Nonadherent marrow mononuclear cells were cultured with M-CSF (10 ng/mL) for 3 days, then the OCL precursors were cultured with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or RANKL in  $\alpha$ -MEM containing 10% FCS for 4 days. The cells were then stained for TRACP. Cells with > 3 nuclei per cell were counted as OCL. The data are shown as mean ± SD (*n* = 3). \**P* < .01 compared with the same treatment of *Trap-Cre(-)/Taf12<sup>fl/-</sup>* marrow. (B) The enriched OCL cell fraction was cultured on dentin slices for 3 days. Then dentin slices were stained with hematoxylin after removing cells. The bone resorption area was counted using a grid and resorption area was evaluated resorption area/total dentin area.

ruffled border in OCLs [40], we examined the effects of loss of TAF12 on autophagic molecule expression in OCLs. We found that syntaxin17 expression, which is involved in autophagosome formation [41], was significantly lower on Western blots (Fig. 82.6) and by

immunohistochemistry of OCLs from TC(+)/Taf12 mice versus TC(-)/Taf12 mice (Fig. 82.7). These results suggest that TAF12 contributes to OCL differentiation, bone resorption, and CTSK secretion in vivo and may be a novel target for blocking bone resorption.



**FIGURE 82.6** Expression levels of cathepsin K, LC3, and syntaxin 17 in osteoclasts derived from *Trap-Cre/Taf12<sup>fl/-</sup>* mouse bone marrow cultures. Osteoclasts were isolated and treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or RANKL in  $\alpha$ -MEM containing 10% FCS for 2 days. The cell lysates were collected and were assayed by Western blotting using cathepsin K, LC3 and STX17 antibodies.



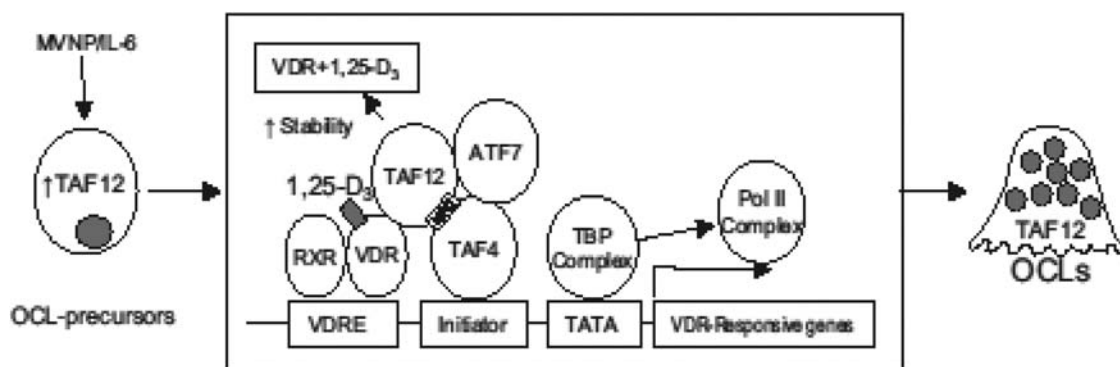
**FIGURE 82.7** Femur sections from *T-Cre(-)/Taf12<sup>fl/-</sup>* and *T-Cre(+)/Taf12<sup>fl/-</sup>* at 8-month-old age stained with TRACP and anti-STX17 ( $\times 40$ ). Inner square ( $\times 40$ ).

## 7. Serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in Paget's patients

We then determined if the increased responsiveness of pagetic OCL precursors to 1,25(OH)<sub>2</sub>D<sub>3</sub> altered vitamin D metabolism, the profile of vitamin D metabolites, Ca, and inorganic phosphate (Pi) by measuring these parameters in the sera of nine patients with PD and 10 age-matched normal healthy volunteers. The concentrations of Vitamin D metabolites, 25OH-D<sub>3</sub>, 24,25(OH)<sub>2</sub>D<sub>3</sub>, and 1,25(OH)<sub>2</sub>D<sub>3</sub>, Ca, and Pi in sera of patients with PD were almost identical to those of age-matched normal healthy volunteers. No abnormality in Vitamin D metabolism was detected in patients with PD. The concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> in sera of patients with PD were  $41.0 \pm 9.1$  pg/mL serum ( $10^{-10}$  M) similar to that of age-matched normal healthy volunteers. These data suggest that there are

adequate levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> in Paget's patients and that these levels should be sufficient to induce OCL formation in the pagetic lesions [42].

Specific treatments for PDB are aimed at increased OCL activity to reduce abnormal bone remodeling. Bisphosphonates (BPs) are currently considered the treatment of choice [43,44]. In particular, the development of newer, more potent nitrogen-containing BPs (N-BPs) has significantly improved treatment outcomes [45]. These compounds show a high binding affinity to hydroxyapatite and increased potency for inhibition of bone resorption [46–48]. They are administered as oral (alendronate and risedronate) or intravenous (zoledronate and neridronate) regimens. All intravenously administered N-BPs can induce an APR, which is characterized by fever, musculoskeletal pain, and other flu-like symptoms, and is treated with analgesics and antipyretics. Medical intervention may be required [49–52]. Although the cause of APR could be attributed to inflammation due to elevated levels of pro-inflammatory cytokines [53,54], interestingly, an observation in 90 postmenopausal women treated with N-BP for osteoporosis revealed: a negative association between circulating 25-hydroxyvitamin D (25(OH)D) levels and the development of APRs [55]. These results suggested that adequate administration of vitamin D preparations to vitamin D-deficient Paget's disease patients decreases the prevalence of common acute adverse events such as APRs following infusion of N-BPs [56]. Previously, less inflammatory VDR gene polymorphisms have also been investigated and may contribute to the reduction of APRs [57]. Adequate vitamin D status may contribute to obtaining a less inflammatory bone phenotype for bisphosphonates that act in PD.



**FIGURE 82.8** Model for 1,25(OH)<sub>2</sub>D<sub>3</sub> hyperresponsiveness of OCL precursors in PD. In this model, expression of the MVNP gene in pagetic OCL precursors increases IL-6, which in turn increases levels of TAF12. TAF12 forms transcription complexes with TAF4 and ATF7. The high levels of TAF12 permit the formation of VDR/TAF12/TAF4 transcription complexes at low levels of receptor occupancy by 1,25(OH)<sub>2</sub>D<sub>3</sub>. VDR-mediated transcription is then enhanced by ATF7. However, because ATF7 does not bind VDR directly; it is unclear if TAF12 is recruited to the CYP24A1 promoter by ATF7 or VDR. It is possible that VDR recruits TAF12 and the TAF12/TAF4–VDR complex and then brings in ATF7 to the VDRE to enhance VDR-mediated transcription. *MVNP*, measles virus nucleocapsid protein; *OCL*, osteoclast; *OCL-pre*, OCL precursors; *RXR*, retinoid receptor; *TBP*, TATA-binding protein; *VDR*, vitamin D nuclear receptor; *VDRE*, vitamin D response element.

## 8. Conclusions

The results presented in this chapter suggest that pagetic OCL precursors are hyperresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub> because of increased levels of TAF12, which acts as a VDR coactivator to enhanced VDR-mediated transcription. A model for the increased 1,25(OH)<sub>2</sub>D<sub>3</sub> responsivity in OCL in PD is shown in (Fig. 82.8). In this model, expression of the MVNP gene in pagetic OCL precursors increases IL-6, which in turn increases levels of TAF12. TAF12 forms transcription complexes with TAF4 and ATF7. The high levels of TAF12 permit the formation of VDR/TAF12/TAF4 transcription complexes at low levels of receptor occupancy by 1,25(OH)<sub>2</sub>D<sub>3</sub>. VDR-mediated transcription is then enhanced by ATF7. These results support the hypothesis that the pathophysiology underlying the increased OCL activity in PD is in part due to increased levels of VDR coactivators that enhance VDR-mediated transcription in OCL precursors and marrow stromal cells exposed to low levels of 1,25(OH)<sub>2</sub>D<sub>3</sub>, these results suggest that PD may represent in part an example of a VDR coactivator disease.

## 9. Summary points

- Osteoclast precursors from Paget's patients are hyperresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub> and express TAF12 that is TATA-Box binding protein-associated factor.
- TAF12 acts as a co-activator of VDR-mediated gene expression, by forming a complex with ATF7 and TAF4 that binds VDR via TAF12 and plays a key role in OCL formation stimulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> in both normals and Paget's patients.

- There is an increased occurrence of APRs to bisphosphonates in Paget's patients with low serum levels of 25OHD

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# Vitamin D metabolism and disorders in companion animals

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## CHAPTER OBJECTIVES

- To advance understanding in the role of vitamin D in skeletal and non-skeletal health in companion animals.
- To provide an overview of congenital and acquired disorders of vitamin D metabolism in companion animals.
- To highlight how vitamin D status can be diagnostically assessed in companion animals.

## 1. Introduction

Vitamin D plays an important role in regulating calcium metabolism and in the development and maintenance of skeletal health of veterinary species. In addition, and paralleling a similar phenomenon in human medicine, there is growing interest in understanding the role vitamin D has on nonskeletal health outcomes in companion animal species [1]. Here, we review vitamin D metabolism in dogs and cats and discuss both congenital and acquired vitamin D disorders. We conclude with an overview of our current understanding of the role vitamin D plays in the non-skeletal health of dogs and cats.

### 1.1 Metabolism and role of vitamin D on skeletal health

The importance of vitamin D on skeletal health has been known for almost a century [2]. In fact, the classic

experiments of Sir Edward Mellanby involved dogs, and through the supplementing of their restricted diet with either linseed or cod liver oil, he demonstrated that cod liver oil, but not linseed oil, could protect dogs from developing rickets [3]. The antirachitic factor in cod liver oil was later discovered to be vitamin D [2]. Although unknown to Sir Edward Mellanby at the time, the heavy reliance of dogs on dietary sources of vitamin D and their failure to produce vitamin D cutaneously meant that their vitamin D-depleted diet was highly effective at inducing rickets in his experimental population.

Veterinary species vary widely in their ability to produce vitamin D cutaneously. Dogs and cats, unlike many other mammals, cannot produce vitamin D cutaneously so are reliant on their diet to obtain vitamin D [4–7]. In contrast, sheep, cattle, and pigs are able to produce vitamin D cutaneously. Both experimental and observational studies in countries where UVB exposure seasonally varies provide clear evidence of the importance of cutaneously derived vitamin D in dogs [7,8]. Dogs and cats are unable to cutaneously produce vitamin D due to the high activity of 7-dehydrocholesterol reductase enzyme that converts 7-dehydrocholesterol into cholesterol. In vitro studies have revealed that total 7-dehydrocholesterol concentrations in the skin of dogs and cats were markedly lower than rats. After sun exposure, there was no change in vitamin D<sub>3</sub> concentrations in dog and cat skin extracts, while there was a 40-fold increase in vitamin D<sub>3</sub> concentrations in rat skin [5]. In cats, an in vivo study demonstrated that the vitamin D status of kittens increases following treatment with a 7-dehydrocholesterol reductase enzyme inhibitor when exposed to ultraviolet light [9]. It is unclear why cats and dogs have a striking failure to cutaneously generate

vitamin D in contrast to almost all other mammals, which have been studied to date; it is widely speculated that their inability to synthesize vitamin D in the skin is related to the consumption of prey containing significant amounts of vitamin D, notably in their liver and adipose tissue, which means there is no physiological requirement for the skin to produce vitamin D [10].

Dietary vitamin D is available in two forms, namely vitamin D<sub>2</sub> (ergocalciferol) and D<sub>3</sub> (cholecalciferol), with most pet foods supplemented with vitamin D<sub>3</sub> (cholecalciferol) [11]. Following intestinal absorption, vitamin D<sub>2</sub> and D<sub>3</sub> enter the circulation and are predominately bound to the vitamin D-binding protein (DBP), with a small percentage also bound to albumin; [12,13] other vitamin D metabolites are also bound in this manner. Less than 1% of vitamin D circulates as free (unbound) and is readily available for cellular utilization [14,15]. It is possible that vitamin D that is bound to the DBP can also exert a biological effect as several target organs of 25(OH)D express megalin, a transmembrane protein that mediates internalization of DBP-bound metabolites. Vitamin D<sub>2/3</sub> are pro-hormones that are subsequently activated by sequential hydroxylation steps by the action of cytochrome P450 (CYP) enzyme family [16]. Vitamin D is first hydroxylated at C25 in the liver to 25-hydroxyvitamin D (25(OH)D), primarily catalyzed by 25-hydroxylases CYP2R1 in the endoplasmic reticulum of the liver and to a lesser extent by CYP27A1 in the liver mitochondria [17,18]. Hydroxylation at C1 $\alpha$  in the proximal tubule of the kidney converts 25(OH)D to the most hormonally active form, 1 $\alpha$ ,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) (calcitriol). Hydroxylation at C1 $\alpha$  occurs in the kidney mitochondria by the action of 1 $\alpha$ -hydroxylase CYP27B1 [19] (see Chapter 4). This enzyme has been detected in other tissues and cell types, and evidence of local production of 1,25(OH)<sub>2</sub>D was a major contributor to identifying extraskeletal roles of vitamin D [20–22] (see Chapter 9). The regulation of CYP27B1 is tightly controlled via parathyroid hormone (PTH) [23–25] and fibroblast growth factor 23 (FGF23) [26], as well as a negative feedback loop whereby 1,25(OH)<sub>2</sub>D acts on itself to suppress CYP27B1 and induce its own catabolism by promoting CYP24A1 [24,27] (see Chapter 8). CYP24A1 can induce C23 and C24 hydroxylation of 1,25(OH)<sub>2</sub>D and 25(OH)D [16,28,29]. C24 hydroxylation of 1,25(OH)<sub>2</sub>D results in a five-step process, which culminates with calcitroic acid being excreted in the bile [30]. C24 hydroxylation of 25(OH)D<sub>3</sub> forms 24,25-dihydroxyvitamin-D<sub>3</sub> (24,25(OH)<sub>2</sub>D<sub>3</sub>). 24,25(OH)<sub>2</sub>D<sub>3</sub> was thought to be an inactive catabolic product of 25(OH)D<sub>3</sub>; however, studies have now shown that it exerts biological activity independent of the vitamin D receptor (VDR) [31–34] (see Chapter 5).

Vitamin D metabolites can also be metabolized via the C3-epimerization pathway. C3-epimerization results in the hydroxyl group at position C3 of the

A-ring of the vitamin D metabolite structure being converted from the alpha to beta orientation, forming stereoisomers of the original metabolite (the only difference between the molecules is the spatial arrangement of the hydroxyl group at C3). All major vitamin D intermediate metabolites are susceptible to epimerization in this way; the process is irreversible and is independent on the presence of a hydroxyl group at the C1 $\alpha$  and C25 positions; [35] and epimers can be further metabolized by hydroxylases at C1 $\alpha$  and C25 as in the standard pathway [36–38]. This means that, not only can 25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D all be epimerized to 3-epi-25(OH)D, 3-epi-1,25(OH)<sub>2</sub>D, and 3-epi-24,25(OH)<sub>2</sub>D, respectively, but 3-epi-25(OH)D can be C1 $\alpha$  and C24 hydroxylated by their respective CYP enzymes [37–40]. The enzyme responsible for C3-epimerization has yet to be identified, but the process can occur in a range of extrarenal tissues and cell types, and it employs enzymes distinct from those classically involved in vitamin D metabolism [41–43] (see Chapter 6).

The principal role of 1,25(OH)<sub>2</sub>D, acting alongside PTH and calcitonin, is to maintain circulating calcium concentrations within a tight reference range [1,44]. 1,25(OH)<sub>2</sub>D exerts its actions on target cells and tissues following binding to the VDR, which is widely expressed in most canine tissues [45]. The VDR-1,25(OH)<sub>2</sub>D complex then heterodimerizes with the retinoic acid receptor, retinoid X receptor (RXR). This complex exerts genomic actions as a transcription factor to regulate target genes that contain a vitamin D response element in their promoter, which then influence numerous physiological processes [2] (Chapters 10–13). Alternatively, 1,25(OH)<sub>2</sub>D can bind to the plasma membrane VDR to induce rapid response biological actions, for example, the stimulation of intestinal calcium transport, which are non-genomic actions of 1,25(OH)<sub>2</sub>D [46,47]. Calcium homeostasis is achieved mainly by the ability of 1,25(OH)<sub>2</sub>D to increase intestinal absorption of calcium [46]. 1,25(OH)<sub>2</sub>D can also increase calcium reabsorption in the distal renal tubule and mobilizes the release of calcium from the skeleton in conjunction with PTH during periods of hypocalcemia [48]. When circulating concentrations of calcium decline, increased plasma PTH concentrations lead to an increase in renal CYP27B1 activity, which in turn raises circulating 1,25(OH)<sub>2</sub>D concentrations. The epimeric forms of 1,25(OH)<sub>2</sub>D also appear to have a role in calcium homeostasis. For example, 3-epi-1,25(OH)<sub>2</sub>D<sub>3</sub>, the most biologically active epimer, can suppress PTH secretion to a similar degree as the parent metabolite [35,37,46]. Despite this, 3-epi-1,25(OH)<sub>2</sub>D<sub>3</sub> has low calcemic effects [39,49] and anti-proliferative activity of ~30% of that of the non-epimeric form; [37,38] it also exhibits reduced affinity for binding to the DBP and the

VDR [38,40,41,50,51]. This reduced affinity results in reduced ability to induce calcium transport [52].

## 1.2 Measurement of vitamin D metabolites in veterinary species

Although a wide range of vitamin D metabolites can be measured using a variety of instrumentation and technologies, the primary vitamin D metabolites measured in both human and veterinary clinical cases are 25(OH)D and 1,25(OH)<sub>2</sub>D [53]. 25(OH)D has a half-life of 2–3 weeks and is widely regarded as the most accurate measurement to assess vitamin D status [54]. 25(OH)D serves as a reservoir for the generation of the more biologically active 1,25(OH)<sub>2</sub>D. 25(OH)D can be measured by a wide range of assays including chemiluminescent immunoassay, enzyme immunoassay, radioimmunoassay, high-performance liquid chromatography (HPLC), and the widely considered gold standard technique of liquid chromatography tandem mass spectrometry (LC-MS/MS) [55,56] (see Chapters 48–50).

The accurate quantification of vitamin D metabolites, even of 25(OH)D, can be challenging. The vitamin D pathway is a highly complex and dynamic system involving a number of structurally very similar compounds, which may interfere with analysis; not only that but also the metabolites circulate predominantly bound the DBP and at low concentrations, meaning they are particularly challenging to identify, isolate, and accurately quantify (see Chapter 49).

Immunoassay-based techniques can be integrated into fully automated laboratory systems allowing for rapid analysis in a high-throughput clinical chemistry laboratory setting. They offer good sensitivity for 25(OH)D and require minimal sample volume; however, selectivity continues to be one of their major limitations [57–59]. Cross-reactivity with different vitamin D metabolites, especially 24,25(OH)<sub>2</sub>D, occurs in many of the immuno-based assays. Lack of selectivity between 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>, and unequal cross-reactivity of the two metabolites can cause bias and have a significant impact depending on the sample being analyzed. Furthermore, vitamin D metabolites must be released from the DBP to be measured, which is difficult to achieve in automated immunoassays in which strong organic solvents cannot be used [57,58]. Therefore, samples in which variation in the DBP levels exists (during pregnancy or cases of renal disease, for example) are known to impact on the performance of these assays.

LC-MS/MS is considered the gold standard method for analyzing vitamin D metabolites in veterinary patients [60–62]. This method does not suffer from those limitations outlined before for immunoassays and has

the capability to detect and quantify multiple, highly similar analytes simultaneously within one sample, enabling the profiling of several metabolites of the vitamin D pathway. Using LC-MS/MS facilitates the effective release of vitamin D metabolites from the DBP during sample preparation by the use of strong organic solvents. High levels of selectivity are achieved in LC-MS/MS methods by the chromatographic resolution of individual analytes and detection based on specific mass-to-charge (*m/z*) ratios and fragmentation patterns, meaning that D<sub>3</sub> and D<sub>2</sub> metabolites can be distinguished and quantified individually.

Isomeric and isobaric metabolites such as the C3-epimers complicate the bioanalytical assessment of vitamin D metabolites even by LC-MS/MS. The C3-epimers have the same molecular mass as the non-epimer metabolites, meaning they are not distinguished in the mass spectrometer and many LC-MS/MS assays do not separate them. However, optimization of the LC parameters and the use of specialist LC columns can ensure that sufficient resolving power can be achieved and the C3-epimers can be chromatographically separated for accurate quantitation. Although low levels of 3-epi-25(OH)D have been recently detected in dogs [63], cats have much higher concentrations [64], which, given the unconfirmed biological activity of the C3-epimers, if not resolved from 25(OH)D may overestimate 25(OH)D concentration.

As LC-MS/MS has the capability to assess multiple metabolites from a single sample, assays that include the measurement of other vitamin D metabolites are now being reported. Since 1,25(OH)<sub>2</sub>D has the most impact on calcium homeostasis, there are clinical indications for its measurement, notably in dogs with hypercalcemia caused by calcitriol exposure. A range of assays have been reported for the quantification of 1,25(OH)<sub>2</sub>D, including immunoassays and LC-MS/MS methodologies [56]. Serum concentrations of 24,25-dihydroxyvitamin D, the degradation product of 25(OH)D, have been quantified in dogs and found to be present in higher concentrations than other species [65]. 24,25-Dihydroxyvitamin D can only be measured by LC-MS/MS, which also provides concomitant quantification of 25(OH)D, thus allowing calculation of the 25(OH)D: 24,25-dihydroxyvitamin D ratio, also known as the vitamin D metabolite ratio (VMR).

Free 25(OH)D concentrations have recently been quantified in dogs for the first time using an enzyme-linked immunosorbent assay (ELISA). In 117 healthy dogs, the median and interquartile range of free 25(OH)D concentrations detected was 15.2 (12.5–23.2) pmol/L [63]. Further studies are required to ascertain whether total or free 25(OH)D concentration is the best assessment of vitamin D status in companion animals. This is likely to have important clinical implications.



For example, in people, DBP levels change significantly during pregnancy influencing the concentration of vitamin D metabolites including 25(OH)D [66] highlighting the merits of measuring free 25(OH)D [67].

One of the main challenges regarding vitamin D analysis in veterinary samples is the lack of standardization between laboratories [68]. Harmonization of 25(OH)D testing has been historically challenging but has improved recently through the development of standard reference materials (SRMs) for some metabolites [69] and reference method procedures (RMPs) [70–72] by the Vitamin D Standardization Program (VDSP) and External Quality Assessments (EQAs) such as the Vitamin D External Quality Assessment Scheme (DEQAS). DEQAS, a global quality assurance scheme for laboratories measuring vitamin D metabolites [73], enables participating laboratories to validate and continuously monitor their assay performance in comparison with both National Institute of Standards and Technology (NIST) RMPs and other laboratories using the same method. These programs have considerably reduced the variability between laboratories [68,74]. SRMs will be required for other vitamin D metabolites, and there are currently no EQAs specific for the assessment of vitamin D in veterinary samples (DEQAS assesses the analysis of vitamin D metabolites in human serum samples). This aspect of vitamin D quantification is discussed in greater detail in Chapter 49.

## 2. Dog

### 2.1 Congenital vitamin D disorders

Congenital disorders of vitamin D homeostasis are well described in dogs although they are rarely observed in clinical practice. Similar to the approach taken in classifying human vitamin D metabolism disorders, genetic vitamin D disorders in animals are typically subdivided into three main types [75]. Vitamin D–dependent rickets type 1A (VDDR-1A) refers to animals with CYP27B1 deficiencies, which leads to impaired conversion of 25(OH)D to the most metabolically active vitamin D metabolite 1,25(OH)<sub>2</sub>D [76]. In contrast, vitamin D–dependent rickets type 1B is caused by mutations in CYP2R1 gene, leading to failure of vitamin D to be converted to 25(OH)D [77]. Both type 1A and 1B are inherited as autosomal recessive traits [76]. The main consequences of these disorders are hypocalcemia, which can be severe enough to cause seizures and skeletal abnormalities; generalized skeletal pain can also be a feature [75]. The third group of genetic vitamin D disorders is vitamin D–dependent rickets type 2A (VDDR-2A), also called hereditary vitamin D–resistant rickets (HVDRR), which involves mutations in the VDR gene. In this rare

condition, hypocalcemia, secondary hyperparathyroidism, and increased concentration of 1,25(OH)<sub>2</sub>D are typical biochemical changes alongside skeletal changes and accompanying pain, which is consistent with rickets [78]. Alopecia may also be a feature of VDR gene mutations. Treatment is challenging and based around high-dose calcium and 1,25(OH)<sub>2</sub>D supplementation. VDDR-2A has been reported in a Pomeranian dog [78].

Provisional diagnosis of these congenital vitamin D disorders is usually based on compatible clinical signs in young patients, who have a dietary history, which demonstrates adequate vitamin D intake. Definitive diagnosis can be more challenging although a genetic test for VDDR-2A is available for Pomeranian dogs (Laboklin, UK).

### 2.2 Acquired vitamin D disorders—deficiency

Acquired vitamin D disorders can result from either an excess or deficiency of vitamin D. An important cause of vitamin D deficiency in companion animals is the consumption of a diet, which is deficient in vitamin D. The European pet food industry (FEDIAF) nutritional guidelines state that the minimum recommended vitamin D allowance for adult dogs is 55.2 IU and for adult cats 25 IU per 100g dry matter. One study demonstrated that 25(OH)D concentration was highly variable, including some instances of hypovitaminosis D, in healthy dogs fed a variety of different commercial diets, possibly implying that some commercial diets might contain inadequate quantities of vitamin D [79]. However, a recent study demonstrated that the majority of proprietary dog foods tested contained vitamin D concentrations within the manufacturers' stated range [80]. Consequently, most cases of hypovitaminosis D occur in dogs and cats fed improperly prepared homemade diets [79,81–84]. The hypovitaminosis D state may result in hypocalcemia, secondary hyperparathyroidism, and possible skeletal abnormalities, typically rickets in young animals and osteomalacia in older dogs [85]. In rickets, the classical skeletal changes include impaired mineralization of physeal and epiphyseal cartilage with lesions typically involving the fastest growing bones such as the radius, tibia, metacarpals, and metatarsals. Clinically, this manifests as a stiff, lame gait, deformed limbs, pain on palpation of bones, and muscle weakness. Seizures may also occur due to hypocalcemia [75]. On radiographic and postmortem examination, typical changes include widening of the physeal growth plate, metaphyseal flaring, poor skeletal mineralization, and potentially pathological fractures [75].

Low vitamin D status has been reported in dogs with gastrointestinal disorders, especially in dogs with a

protein losing enteropathy (PLE) [86,87], as well as dogs with exocrine pancreatic insufficiency [88], acute pancreatitis [89], and liver disease [90].

### 2.3 Acquired vitamin D disorders—excess

Vitamin D excess and associated hypercalcemia can occur in dogs through the consumption of diets containing disproportionately high concentrations of vitamin D. This typically occurs as a consequence of inadvertent consumption of vitamin D containing rodenticides or medications, or through the administration of inappropriately vitamin D-enriched commercial diets [91–94]. As with vitamin D minimum values, the (FEDIAF) nutritional guidelines also state the safe maximum values and European legal limit of vitamin D that canine commercial diets should contain is 320 IU/100g DM (EU legal limit 227 IU/100g DM). Homemade and commercial raw diets have been shown to contain vitamin D concentrations above the recommended maximum [95]. Although dogs can tolerate oral vitamin D doses above the recommended allowance of 55.2 IU/100g DM without major ill effect [96], significant overfortification of foodstuff with vitamin D can lead to debilitating hypercalcemia [97].

Hypervitaminosis D secondary to rodenticide consumption is well recognized and, unfortunately, is likely to be an increasing problem as some rodenticide manufacturers switch to vitamin D-containing products [93,98]. Consumption of vitamin D-containing medication is also an increasingly well-recognized cause of hypervitaminosis D in companion animals. This can arise due to the ingestion of oral vitamin D supplements or unintentional consumption of ointments containing vitamin D analogs, now widely prescribed for psoriasis treatment [99–101]. There have been reported cases of dogs developing hypervitaminosis D as a consequence of licking owner's skin covered in topical ointments containing 1,25(OH)<sub>2</sub>D or vitamin D analogs or through consumption of detached 1,25(OH)<sub>2</sub>D-containing skin plaques [102,103].

Clinical disease occurs in hypervitaminosis D states when vitamin D metabolite concentrations become sufficiently increased to cause hypercalcemia, resulting in clinical signs such as polydipsia, polyuria, lethargy, and inappetence. Acute renal failure and long-term complications caused by widespread soft-tissue mineralization can occur in the most severely affected individuals [93]. Two dogs diagnosed with hypervitaminosis D had serum 25(OH)D concentrations around 400 nmol/L (reference range 17–140 nmol/L) when measured in DEQAS accredited laboratory [104]. Where there is known exposure to potentially toxic doses of vitamin D, immediate treatment involves induction of emesis

followed by administration of activated charcoal, in conjunction with supportive and symptomatic care, typically involving intravenous fluid therapy [93]. Intravenous lipid emulsion has been recently reported as a method to successfully lower serum 25(OH)D concentrations in a dog with vitamin D toxicity [105]. Both experimentally and clinically, bisphosphonates have been successfully used to treat hypervitaminosis D, with intravenous pamidronate or zoledronate the most widely utilized [54,106]. While bisphosphonates are effective and generally well tolerated in dogs [107], complications such as osteonecrosis are increasingly well recognized [108,109]. The lipid-soluble properties of vitamin D can lead to sequestration of the vitamin into fat deposits in the body. Consequently, it can take several months for the serum 25(OH)D concentrations to return to within the reference interval following a hypervitaminosis D episode [98].

Endogenous overproduction of active vitamin D metabolites is another mechanism for the development of hypervitaminosis D. This classically occurs in patients with granulomatous diseases where a dysregulated immune response results in the excessive production of 1,25(OH)<sub>2</sub>D from 25(OH)D, typically by macrophages, without the regulation of negative feedback. This syndrome has been reported in dogs with sterile granulomatous lymphadenitis [110], granulomatous inflammation following placement of a biological implant [111], *Angiostrongylus vasorum* infections [112] and *Mycobacterium avium* subspecies *hominissuis* infection [113]. Excessive production of 1,25(OH)<sub>2</sub>D has also been postulated to be important in driving hypercalcemia in dogs with autoimmune diseases such as immune-mediated polyarthritis [114]. Successful treatment of the underlying condition typically resolves the increases in systemic 1,25(OH)<sub>2</sub>D concentrations and associated hypercalcemic state [114].

### 2.4 Role of vitamin D on non-skeletal health in dogs

The vital role of vitamin D in the maintenance of bone health and calcium homeostasis has been widely acknowledged since the discovery of vitamin D nearly a century ago. Since the more recent discovery of VDR expression in tissues beyond the intestines, kidney, and bone, many studies have investigated the non-skeletal health benefits of vitamin D in both humans and dogs [1,115].

#### 2.4.1 Gastrointestinal diseases

In dogs with PLE, total hypocalcemia is not an uncommon biochemical abnormality, and historically, this has been attributed to a reduction in protein-

bound calcium, secondary to hypoalbuminemia [116,117]. However, the wider availability of ionized calcium assays has generated a greater awareness that many dogs with a PLE have reductions in ionized as well as protein-bound calcium [118]. While a blunted PTH response due to concurrent hypomagnesemia has been postulated to be an important driver of hypocalcemia in some cases of PLE [119], there is a growing consensus that low concentrations of 25(OH)D play an important role in the development of the hypocalcemic state associated with PLE in dogs [86,120]. A positive correlation was identified between 25(OH)D concentrations, serum albumin, and ionized calcium levels in dogs with inflammatory bowel disease (IBD) in one study [86]. This study also demonstrated that dogs with PLE had markedly lower 25(OH)D concentrations compared with a healthy control population, dogs with intestinal inflammation who were normoalbuminemic and hospitalized dogs without gastrointestinal signs. Furthermore, serum 25(OH)D concentration has been found to be negatively correlated with duodenal histopathology scores [121] and inversely correlated with canine IBD activity index [86]. Similarly, dogs with a chronic inflammatory enteropathy (CIE) and low serum 25(OH)D concentrations had higher canine chronic enteropathy clinical activity index scores compared with CIE dogs with normal serum 25(OH)D concentrations [121]. In some dogs with a PLE, the hypovitaminosis D and secondary hypocalcemia can be so severe that neurological signs, including seizures, can be a significant clinical complication of the intestinal disease [122,123]. Additionally, the severity of the hypovitaminosis D state has been shown to correlate with adverse clinical outcomes in dogs with a PLE [120,124].

The cause of hypovitaminosis D in PLE is likely to be multifactorial. Malabsorption of fat-soluble vitamin D (especially in dogs with lymphangiectasia) is widely considered to be the most important cause; however, if this was the sole etiology, malabsorption of other fat-soluble vitamins, such as vitamin K, might be expected to occur simultaneously [119]. Although this has not been specifically investigated, significant vitamin K deficiency would be a rare complication of PLE given the absence of coagulopathies caused by a deficiency in vitamin K-dependent clotting factor although dogs with PLE have been described as hypercoagulable [125].

Other factors that may contribute to the depletion of vitamin D in PLE patients include ongoing systemic inflammation, reduced dietary intake of vitamin D, and increased vitamin D metabolism. Additionally, 1,25(OH)<sub>2</sub>D has immunomodulatory effects, which may support mucosal health [126]. Due to this immunomodulatory role, it is also possible that patients with PLE develop this disease in part due to their 25(OH)D deficiency [67]. A human study demonstrated that

women who had low 25(OH)D levels were at an increased risk of the development of Crohn's disease [67]. Finally, magnesium is required for the hydroxylation step necessary to produce 1,25(OH)<sub>2</sub>D in the renal tubules. Therefore, concurrent hypomagnesemia in dogs with PLE may consequently reduce the amount of 1,25(OH)<sub>2</sub>D available [119]. Protocols for correction of hypovitaminosis D associated with chronic enteropathy have not been well established in human or veterinary medicine. Although parenteral administration of calcitriol would be logical, due to the concerns about impaired gastrointestinal absorption of fat-soluble vitamins, in people with IBD, enteral supplementation is often utilized and is beneficial [127]. The optimal dose of calcitriol and the duration of treatment are also uncertain [86].

#### **2.4.2 Pancreatic and hepatic diseases**

Dogs with pancreatic and hepatic disorders have also presented with low serum concentrations of 25(OH)D. Dogs with weight loss and exocrine pancreatic insufficiency (EPI) were found to have significantly lower serum 25(OH)D concentrations than dogs with EPI and stable weight [88]. Additionally, concentrations of three fat-soluble vitamins (A, E, and D) remained reduced in dogs with EPI even after pancreatic enzyme supplementation [88]. The reason for this is uncertain, hypothesized explanations include; long-term dietary fat malabsorption, which is refractory to pancreatic enzymes supplementation, depletion of stored vitamin D due to the loss of adipose tissue associated with weight loss, or ongoing reduced dietary intake secondary to the EPI. Dogs with acute pancreatitis had lower 25(OH)D status than healthy dogs [89]. In addition, dogs with acute pancreatitis that survived had significantly higher serum 25(OH)D concentrations than dogs who died [89]. This positive correlation between reduced vitamin D status and prognosis has also been identified in human studies [128,129]. Vitamin D deficiency and rickets has occasionally been reported in dogs with liver diseases likely as a consequence of impaired intestinal absorption of vitamin D [90]. Vitamin D status has been negatively linked to disease severity in dogs with liver disorders [130]. Serum concentrations of 25(OH)D increased following the surgical correction of a congenital extrahepatic portosystemic shunt [131].

#### **2.4.3 Infectious diseases**

Vitamin D status has been found to be lower in patients with a wide variety of infectious diseases. Dogs with clinically active leishmaniasis [132], blastomycosis [133], babesiosis [134], and *Spirocerca lupi* [135] infections had lower 25(OH)D status than healthy dogs. Similarly, cats with mycobacteria and feline immunodeficiency virus infections had lower 25(OH)D concentrations than



healthy cats [136,137]. A negative relationship between 25(OH)D concentrations and disease severity has also been reported in dogs with babesiosis and *S. lupi* infections [134,135]. Serum concentrations of 25(OH)D have been found to decline in dogs, which developed clinical leishmaniasis [138]. Vitamin D status was also lower in shelter dogs with infectious respiratory disease complex compared with healthy shelter dogs [139].

#### **2.4.4 Immune-mediated diseases**

The role of vitamin D in immune-mediated and inflammatory diseases was explored, in part, due to the identification of the VDR on the surface of many cell types, including leukocytes [140]. Consequently, dogs with a range of immune-mediated diseases, including immune-mediated hemolytic anemia, thrombocytopenia, and polyarthritis, have been found to have altered vitamin D homeostasis [141] and reduced concentrations of 25(OH)D. Additionally, low serum 25(OH)D concentrations were also predictive of a poorer clinical outcome in dogs with immune-mediated diseases [141]. Dogs with acute polyradiculoneuritis had lower circulating 25(OH)D concentrations than dogs with idiopathic epilepsy [142]. Interest in the relationship between autoimmune diseases and vitamin D has been fueled by the growing evidence that vitamin D metabolites can influence the canine immune cell function and phenotype *ex vivo*, typically switching innate immune cells from a pro-inflammatory to a more anti-inflammatory response [143,144]. For example, a recent *in vitro* study using blood from healthy shelter dogs identified that the concentrations of tumour necrosis factor- $\alpha$  significantly decreased, while interleukin-10 significantly increased, following incubation with 1,25(OH) $_2$ D [145].

#### **2.4.5 Endocrine diseases**

Dogs with naturally occurring hypercortisolism have been found to have 25(OH)D concentrations lower than healthy dogs [146]. The cause of this decrease is incompletely understood but may be related to increased urinary loss of vitamin D metabolites or due to increased degradation of 25(OH)D.

#### **2.4.6 Cardiovascular diseases**

Circulating 25(OH)D concentrations were significantly lower in dogs with congestive heart failure compared with unaffected dogs even though the two groups had a similar dietary vitamin D intake [147]. 25(OH)D concentrations were also found to be lower in dogs with chronic valvular disease stage B2 and C/D disease than dogs with stage B1 disease [148].

#### **2.4.7 Oncology**

The connection between vitamin D status and neoplasia has been extensively investigated in human medicine, with one study finding that serum 25(OH)D concentrations greater than 40 ng/mL resulted in a reduced risk of rectal, colonic, and breast cancer [149]. Several studies have shown that vitamin D metabolites have anti-proliferative effects on canine cancer cell lines *in vitro* [150–152]. Vitamin D status of dogs with cancer has frequently been reported to be lower than control populations. For example, dogs with mast cell tumors had lower 25(OH)D concentrations than healthy dogs despite having no significant differences in vitamin D oral consumption [153]. Dogs with three different types of cancer (mast cell tumor, lymphoma, and osteosarcoma) all had altered vitamin D metabolism compared with the healthy control population [154]. Another study found that relative risk of cancer in dogs increased with decreasing 25(OH)D concentrations [155]. Dogs with B cell lymphoma were found to have lower plasma concentrations of 25(OH)D than healthy dogs [154]. However, other studies have failed to find a difference in vitamin D homeostasis in dogs with and without cancer including the observation that 25(OH)D concentrations were not significantly different between dogs with an osteosarcoma and control dogs [156].

#### **2.4.8 Urinary tract disease**

In dogs with chronic kidney disease (CKD), lower levels of serum 25(OH)D have been identified [157,158]. In addition, low concentrations of 1,25(OH) $_2$ D have also been reported in dogs with CKD. This is thought to be due to a combination of lower availability of 25(OH)D, reduced CYP27B1 (1 $\alpha$ -hydroxylase) activity due to renal damage, and urinary loss of vitamin D [158,159]. The decline in 1,25(OH) $_2$ D concentration is considered important in the development of secondary hyperparathyroidism in CKD, leading to interest in the potential therapeutic merits of 1,25(OH) $_2$ D supplementation in companion animals with renal failure [159,160]. One unpublished study found that judicious administration of 1,25(OH) $_2$ D may confer a survival benefit in dogs with IRIS stage 3 and 4 providing calcium, phosphate, and PTH can be closely monitored [161]. A recent study found that administering an extended release form of 25(OH)D to dogs with CKD resulted in an increase in vitamin D metabolites, including 1,25(OH) $_2$ D, while avoiding hypercalcemia [162]. However, the benefits of long-term 25(OH)D supplementation on the progression of CKD, and the patient's quality of life, remained undetermined in this study [162].



Vitamin D homeostasis disorders may increase the risk of other renal disorders, with a recent study suggesting that altered vitamin D catabolism may predispose some dogs to the development of calcium oxalate uroliths [163]. In dogs with non-azotemic protein losing nephropathy (PLN), a significant positive correlation between serum albumin and concentrations of both 25(OH)D and 24,25(OH)<sub>2</sub>D was observed [164]. Additionally, dogs with PLN had significantly lower serum 25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D concentrations compared with the control population [164].

#### 2.4.9 Inflammation

Several studies have shown an inverse relationship between biomarkers of inflammation and vitamin D status in dogs. For example, serum 25(OH)D concentrations in dogs diagnosed with a CE have been shown to negatively correlate with circulating neutrophil, monocyte, and serum pro-inflammatory cytokines [165]. Furthermore, two studies have observed that vitamin D status in dogs with a CE negatively correlated with severity of intestinal inflammation [121,165]. Dogs with blastomycosis also had a negative relationship between vitamin D status and neutrophil counts [133], and CRP was significantly lower in a population of healthy dogs with a serum 25(OH)D concentration above 100 ng/mL compared with dogs with a 25(OH)D concentration below 100 ng/mL [155]. However, the widely reported negative association between vitamin D status and inflammation is not an absolute consensus, with one study reporting an increase in both C-reactive protein and 25(OH)D concentrations in racing sled dogs [65].

#### 2.4.10 Dermatology

Serum 25(OH)D concentrations in dogs with atopic dermatitis were not significantly different from healthy dogs [166]. However, atopic dogs that responded well to prednisolone had higher serum 25(OH)D concentrations [166]. Oral vitamin D treatment has been shown to improve clinical outcomes in an atopic dermatitis treatment trial with an increase in serum 25(OH)D correlating to a reduction in pruritus and skin lesions [167]. In this study, paricalcitol, a VDR agonist, was also trialed in a small number of dogs; however, unacceptable side effects including hypercalcemia and polyuria/polydipsia resulted in withdrawal of treatment [167].

#### 2.4.11 Critical care

Several studies have identified a relationship between low vitamin D status and adverse clinical outcomes in hospitalized dogs. Vitamin D status in critically ill dogs that survived was found to be significantly higher than in dogs that died [168]. Critically ill dogs and dogs with sepsis had significantly lower serum 25(OH)D

concentrations compared with healthy control dogs, and 25(OH)D concentration was an independent predictor of in-hospital and 30-day survival [169]. Experimental induction of inflammation is known to reduce 25(OH)D concentrations in dogs [170].

### 3. Cat

Feline VDDR-1A has been reported [76,171], while type 1B has been reported in a cat [77]. The disorders can be managed, with varying degrees of success, mainly through 1,25(OH)<sub>2</sub>D supplementation. VDDR-2A has been reported in the veterinary literature in cats [172–174]. Experimentally, the dysfunction of the vitamin D receptor in VDDR-2A has been proven by the inability of skin fibroblasts, which express VDR, to effectively bind 1,25(OH)<sub>2</sub>D [172,174]. Additionally, entire DNA sequencing of the feline CYP27B1 gene has also been described as a means of diagnosing VDDR-1A [77]. Low vitamin D status has been reported in cats with gastrointestinal disorders (compared with healthy controls) [175].

Vitamin D excess and associated hypercalcemia can occur in dogs through the consumption of diets containing disproportionately high concentrations of vitamin D. This typically occurs as a consequence of inadvertent consumption of vitamin D containing rodenticides or medications, or through the administration of inappropriately vitamin D-enriched commercial diets [91,93,176,177]. Historically, increased concentrations of vitamin D have been observed in some cat foods, which has been attributed to their high content of oily fish [176,177]. In cases of hypervitaminosis D, acute renal failure and long-term complications caused by widespread soft-tissue mineralization can occur in the most severely affected individuals [93,176,177]. As with vitamin D minimum values, the FEDIAF nutritional guidelines also state the safe maximum values and European legal limit of vitamin D that canine and feline commercial diets should contain: for cats, a maximum of 3000 IU/100 g DM is safe for all feline life stages (EU legal limit 227 IU/100g DM).

#### 3.1 Role of vitamin D on non-skeletal health in cats

Low vitamin D status has been observed in numerous feline infectious diseases including blastomycosis [178], *Mycobacterium* infection [179], feline infectious peritonitis [180], Toxoplasmosis [180], *Nocardia* infection [181], Cryptococcosis [180], feline immunodeficiency virus [137], and rhinitis caused by *Actinomyces* [180].

In addition, many cats with cholestatic liver disease, especially hepatic lipidosis, have been reported to have low serum 25(OH)D levels [182]. In cats, both 25(OH)D and a C-3 epimer of 25(OH)D were lower in cats with cardiomyopathy compared with healthy cats, although, when patient age was taken into account, this did not reach statistical significance since older cats had significantly lower 25(OH)D concentrations [183]. Hospitalized cats with neutrophil counts above the reference range had lower serum 25(OH)D concentrations than cats with neutrophil counts below the upper limit of the reference range [184].

A study of hospitalized cats found that cats, which died within 30 days of initial assessment, had significantly lower 25(OH)D than cats that survived [185]. Further analysis revealed that the relationship between vitamin D status and mortality risk was not linear, as cats within the lowest 25(OH)D tertile had a significantly higher mortality risk than cats in the upper or middle tertile [185]. Low vitamin D status has also been associated with anemia in hospitalized cats [186].

The decline in 1,25(OH)<sub>2</sub>D concentration is considered important in the development of secondary hyperparathyroidism in CKD, leading to interest in the potential therapeutic merits of 1,25(OH)<sub>2</sub>D supplementation in companion animals with renal failure [159,160,187].

#### 4. Conclusions and future directions

While numerous studies have linked low vitamin D status to adverse non-skeletal health outcomes in cats and dogs, there is still significant uncertainty over the importance of vitamin D in shaping clinical outcomes in non-skeletal disorders. The optimal way to supplement vitamin D also remains uncertain, in terms of both the route of administration (enteral vs. parenteral) and the vitamin D metabolite of choice (25(OH)D, 1,25(OH)<sub>2</sub>D or cholecalciferol). The relationship between low 25(OH)D concentrations and the development or outcomes of non-skeletal disorders cannot be considered to be invariably causative and may, in fact, be due to reverse causation. For example, serial measurements of circulating 25(OH)D concentrations in experimental and human patients have shown that inflammation and infections can result in lower vitamin D status [188–190]. A recent study that longitudinally tracked both markers of inflammation and 25(OH)D concentrations in dogs undergoing elective surgery have recently shown that the postoperative increase in acute phase proteins is mirrored by modest declines in total, but not free, serum 25(OH)D concentrations [191]. To disentangle the relationship between vitamin D and non-

skeletal health outcomes, human-focused studies have embraced Mendelian randomization approaches as well as examining the non-skeletal health benefits of long-term vitamin D supplementation in large-scale clinical trials [192,193]. Mendelian randomization techniques use genetic variation as a natural experiment to investigate the causal relations between potentially modifiable risk factors and health outcomes in observational data [193]. This approach has allowed the health consequences of genetically determined low lifetime vitamin D status to be studied in large human populations. Human Mendelian randomization approaches have been highly informative in strengthening the link between low vitamin D status and development of diseases such as multiple sclerosis [194,195]. The complementary approach of investigating the non-skeletal health benefits of vitamin D through supplementation trials, despite their complexities and caveats, is also being highly informative in the human healthcare setting. For example, recent large trials have not shown any clear benefit in reduction of incidence of cardiovascular or cancer [196–198]. However, trials are revealing that vitamin D supplementation can be helpful in respiratory tract disorders, notably in patients with low 25(OH)D concentrations [199,200], and in improving survival times in patients diagnosed with cancer [201,202]. The global COVID-19 pandemic caused by SARS-CoV-2 has stimulated a flurry of interest into the role of vitamin D in both the treatment and prevention of this infection, with some studies postulating that a depleted vitamin D status could be associated with a worse outcome [203]. While these approaches may be challenging to undertake in the veterinary sector, long-term studies of healthy animals of known vitamin D status are necessary to further define the relationship between 25(OH)D concentrations and non-skeletal health outcomes in dogs and cats.

#### 5. Summary points

- Vitamin D metabolism disorders are a significant cause of morbidity and mortality in dogs and cats.
- The importance of vitamin D in the development and maintenance of skeletal health is well established in companion animals. There is a growing awareness of the potential role vitamin D may play in non-skeletal health in dogs and cats.
- The growth in vitamin D metabolite assays, which have been validated for use in companion animal samples, has greatly facilitated the rapid diagnosis of vitamin D disorders in dogs and cats.
- Both vitamin D deficiency and vitamin D excess disorders are well described in dogs and cats.

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## Further reading

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# Overview of vitamin D actions in cancer

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## OBJECTIVES

- To introduce vitamin D and vitamin D receptor in cancer in a historical context.
- To provide insights into processes underlying vitamin D effects on cancer cells.
- To present developments in combinations of vitamin D with other therapies.
- To summarize the impact of vitamin D metabolism and tumor resistance.

## 1. Introduction

The seco-steroid hormone 1,25-dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>] is the most potent metabolite of vitamin D<sub>3</sub> and is an important regulator of calcium homeostasis and bone metabolism via actions in intestine, bone, kidney, and parathyroid glands. 1,25(OH)<sub>2</sub>D<sub>3</sub> exerts its effects via an intracellular receptor that is a member of the steroid hormone receptor family. Throughout the past decades, it has become evident that the vitamin D receptor (VDR) is not limited to cells and tissues involved in regulation of calcium and bone metabolism but is also present in a wide variety of other cells and tissues including cancer cells of various origins. This has led to a growing number of studies on the role of vitamin D in tumor cell growth regulation, treatment of cancer, and development of potent synthetic vitamin D analogs. The anticancer action of vitamin D may not only be limited to the cancer cells itself but also target other cells

in the tumor such as the stromal cells (fibroblasts, adipocytes, and immune and endothelial cells). Various specialized chapters will discuss in detail the effect of vitamin D on specific cancer types (see Chapters 85–92 in this section) and the development of analogs. In this chapter, our goal is to provide an overview of the history and current state of knowledge of the field. We will address several areas: recent developments in studies of vitamin D and cancer, regulation of tumor cells, possible mechanisms, and clinical applications. Since the field has become so vast of course, we could not cite all of the relevant papers, and the reader is referred to the specialized chapters on the various cancers that follow this chapter for more detail.

Note by the authors: We covered a broad range of topics and scientific literature. We realized that it is impossible to include and cite all literature available. Therefore, the reference list should be considered as incomplete, and it is expected that additional references can be found by searching on the various topics discussed. By no means, we had any prejudice in our selection of citation of particular studies.

## 2. Vitamin D and cancer

### 2.1 Vitamin D receptor

As exemplified in Table 84.1, the VDR has been demonstrated in a broad range of tumors and malignant cell types. VDR level is increased in ovarian carcinoma compared with normal ovarian tissue [1]. For colon and breast cancer cells, an inverse relationship between VDR level and degree of differentiation has been described by some investigators [2,3]. For colorectal cancer, it was shown that VDR expression is associated with

**TABLE 84.1** VDR in tumors and malignant cell types.

Basal cell carcinoma	Myeloid leukemia
Breast carcinoma	Multiple myeloma
Bladder cancer	Osteogenic sarcoma
Cervical carcinoma	Ovarian carcinoma
Colonic adenocarcinoma	Neuroblastoma
Colorectal carcinoma	Non-Hodgkin's lymphoma
Gall bladder carcinoma	Pancreatic carcinoma
Glioblastoma	Parathyroid adenoma
Kaposi sarcoma	Pituitary adenoma
Lung carcinoma	Prostate carcinoma
Lymphocytic leukemia	Renal cell carcinoma
Malignant B cell progenitors	Squamous cell carcinoma
Malignant melanoma	Transitional cell bladder carcinoma
Medullary thyroid carcinoma	Uterine carcinosarcoma

the degree of tumor differentiation [4] and with a more favorable prognosis [5]. Accordingly, VDR expression in colon tumor stromal fibroblasts predicted a favorable clinical outcome [6]. This is an important aspect of the anticancer actions of vitamin D: interacting with surrounding stromal cells including cells of the immune system and not only with the cancer cells. In pancreatic cancer, the VDR regulates transcription of pancreatic stellate cells, which results in stromal remodeling, which leads to reduced tumor volume and increased chemotherapeutic response [7]. In hepatocellular carcinoma, SQSTM1 (p62) protein was found to act as a negative regulator of liver inflammation and fibrosis through VDR signaling in hepatic stellate cells [8].

A VDR immunoreactivity score showed an increase in VDR in breast carcinoma specimens compared with normal breast tissue, but no clear relation with proliferative status could be assessed [9]. A later study by the same group showed that VDR expression is not a prognostic factor for breast cancer, but the strong VDR immunoreactivity in the breast cancer specimens supports the evidence that it may be a target for intervention [10]. Also in other studies, no associations between VDR concentration and clinical and biochemical parameters of breast cancer were found [11–13]. These outcomes could be the result of the fact that in clinical human breast tumor samples, variable expression of the VDR was found in different cohorts [14].

A phenomenon that should be taken into account is the intratumor heterogeneity. Single-cell sequencing techniques demonstrated that expression of genes may vary within a tumor, which may explain differences in responses to treatments [15,16]. Novel technological developments such as spatiotemporal single-cell sequencing will provide sequence data in relation to the location in the tumor and help understanding intratumoral heterogeneity [17]. This will also provide

insight into the relation between cancer cells, immune cells, and stroma within the tumor. These are all three targets for vitamin D, and it is conceivable that for VDR expression and vitamin D action, intratumoral heterogeneity is also important. Further illustration of the complex and potentially nonlinear relationship between VDR levels and cancer-related outcomes is shown by the report that both VDR high and VDR low groups show a reduced regression-free survival compared with the VDR normal group [18].

Albeit that the association studies on VDR expression and predictive and/or prognostic characteristics for cancer are so far not conclusive, depending also on other features such as VDR functionality or 25(OH)D levels, the widespread distribution of the VDR in malignant cells indicates that regulation of cancer cell function is an action of 1,25(OH)<sub>2</sub>D<sub>3</sub> and provides a molecular basis for the VDR and vitamin D level association studies. An interesting observation has put the VDR in relation to cancer in another perspective. It was shown that VDR can function as a receptor for the secondary bile acid lithocholic acid [19]. This compound is hepatotoxic and a potential enteric carcinogenic. Interestingly, binding of both lithocholic acid and vitamin D to the VDR results in induction of CYP3A, the enzyme that detoxifies lithocholic acid in the liver and intestine [19,20]. It is postulated that vitamin D and lithocholic acid, by binding to the VDR, activate a feed-forward catabolic pathway that increases CYP3A expression, leading to detoxification of carcinogenic bile acids. In addition to the option that other ligands than vitamin D may activate VDR, a recent mechanism brings forward that vitamin D metabolites may activate another nuclear receptor (LXR) [21]. This brings in the field of vitamin D metabolism beyond or besides CYP24A1 action and understanding their target tissue/tumor type-specific production, stability, and levels. The development in analytical tools, e.g., LC-MS/MS, allows more in-depth analyses of vitamin D metabolism, which may deliver a broad spectrum of metabolites [22].

Although cellular effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> traditionally have been attributed to activation of the nuclear VDR, over the years research has been performed to identify a membrane 1,25(OH)<sub>2</sub>D<sub>3</sub> receptor. Currently, protein disulphide isomerase family member 3 (PDIA3, or ERp57, or 1,25D<sub>3</sub>-MARSS) is the most likely candidate. For more detailed discussion on PDIA3, see 2020 Viewpoint article by Zmijewski and Carlberg [23].

### 2.1.1 Epidemiology

The first to document an association of cancer mortality with sun exposure and latitude was Hoffman in 1915 [24]. Later studies in 1980 by Garland et al. [25] provided additional data showing that death rates from colon cancer tended to increase with increasing latitude and

decreasing sunlight. The sunlight/ecological concept is discussed in [Chapters 56 and 85](#). Later, more direct evidence about a correlation between vitamin D concentration and colon cancer came from the inverse relationship between levels of serum 25-hydroxyvitamin 25(OH)D and the incidence of colon cancer [26,27]. In a metaanalysis, Gorham et al. [28] estimated that an increase of 84 nmol/L (33 ng/mL) in serum 25(OH)D level would lead to a 50% reduction in the incidence of colon cancer. A study of National Health and Nutrition Examination Survey III (NHANES III) data also found an association between 25(OH)D concentration and colorectal cancer mortality. Individuals with a 25(OH)D level over 80 nmol/L (32 ng/mL) had a 75% lower risk of death from colorectal cancer than those with lower levels of 25(OH)D. A concentration over 95 nmol/L correlated with a 55% reduction in colon cancer risk compared with those with a level below 40 nmol/L [29]. Several studies confirmed that a higher concentration of vitamin D was associated with lower colon cancer incidence and patients have a better overall survival [30].

From the NHANES III study, it was reported that women with a serum 25(OH)D concentration of more than 62 nmol/L had a 75% decrease in mortality due to breast cancer [29]. From two other studies, the authors concluded that there was a 58% lower risk of breast cancer in women with a 25(OH)D concentration of more than 95 nmol/L compared with women with levels lower than 37.5 nmol/L [31,32]. In a metaanalysis, 1750 women were stratified into five groups of 25(OH)D concentrations ranging from high to low, and this showed a clear dose–response association [33]. The highest breast cancer rates were found in the group with the lowest 25(OH)D concentration (<32 nmol/L), while the cancer rates were lower at higher levels (>130 nmol/L). Later studies confirmed the relationship between higher 25(OH)D levels and a lower risk for breast cancer progression and mortality [34]. A large Finnish epidemiological study showed an association of low serum 25(OH)D with prostate cancer [35,36]. The incidence of prostate cancer was twice as high in men with a 25(OH)D concentration below 70 nmol/L and 1,25(OH)<sub>2</sub>D<sub>3</sub> levels below 77 pmol/L. A full discussion of the epidemiologic data linking vitamin D and cancer can be found in Chapter 85, and a recent review by Munoz and Grant [37]. It is strongly suggestive that avoiding vitamin D deficiency may be a way to reduce cancer risk and progression [38].

Studies showed that the association between UVB irradiance and prostate cancer incidence depends on the season of irradiance [39], and an Australian study demonstrated a seasonal pattern for testicular germ cell cancer with the highest number of diagnosis in the winter [40]. However, it is difficult to envisage that cancer development and progression, which is often a long-

term, multistage process, depends on a 3-month period like winter within general lowest levels of 25(OH)D. The relationship between sunlight exposure and cancer, especially with respect to vitamin D, had been carefully reviewed earlier by Studzinski and Moore [41]. The dual relationship between sunlight and cancer is of interest and remains the subject of many studies [42–44]. A relation between skin type and prostate cancer has been described [45–47], and an article discussing the skin, sunlight, vitamin D and cancer from an evolutionary perspective has been published [48]. Grant et al. [49] estimated that between 50,000 and 63,000 Americans and between 19,000 and 25,000 adults from the United Kingdom die every year from cancer due to vitamin D deficiency. An analysis of the economic burden due to vitamin D insufficiency from inadequate exposure to solar UVB, diet, and supplements was \$40–56 billion in 2004 versus an economic burden for excess UV irradiation of \$6–7 billion [50]. In Multiple myeloma, lower 25(OH)D levels were associated with higher plasma cell number in the bone marrow, and a high incidence of vitamin D deficiency was found in myeloma patients [51].

The relationship between cancer, diet, calcium intake, and vitamin D has also been addressed in several studies [52–54]. A study on intake of micronutrients suggested that vitamin D and calcium might interact with antioxidants such as vitamin C and E in reducing colorectal cancer risk [55]. It is clear that sunlight exposure, vitamin D intake, and other dietary components such as calcium and fat should be considered as possibly interacting with one another when the relationship between vitamin D and cancer risk is assessed. The data on VDR as bile acid sensor and its postulated role in detoxification provide a direct biological basis for the relation between increased colon cancer and high-fat diets [56] and that colon cancer occurs in areas with higher prevalence of rickets [57]. In addition, mice lacking VDR have been reported to have a higher proliferation rate in the colon [58,59]. A survey of possible mutations in the VDR in osteosarcomas, several other sarcomas, non-small-cell lung cancers, and a large number of cell lines representing many tumor types did not show that mutations or rearrangements in the VDR gene play a role in these cancers [60]. Aspects of sunlight and the epidemiology of vitamin D and calcium will be discussed in greater detail in [Chapters 56 and 85](#).

However, data on the associations between vitamin D and cancer are not consistent. This has been observed in prostate cancer [34]. In a large prospective study by Ahn et al. [61] the hypothesis that vitamin D is associated with decreased risk of prostate cancer was not supported; in contrast, higher circulating 25(OH)D<sub>3</sub> concentrations may be associated with increased risk of aggressive disease. Also in other types of cancer, not



always the same association was found. In breast cancer, similar vitamin D intakes were found in breast cancer patients and control subjects [62]. Moreover, in a mouse model, no relationship was found between dietary intake of a wide range of doses of calcium or vitamin D on carcinogen-induced skin tumors [63].

Also for ovarian cancer, a similar discrepancy was observed. For example, Grant et al. [42,64] reported a strong association between vitamin D levels, geographical latitude, and ovarian cancer mortality, while more recently, Toriola et al. [65] in a case–control study with the Finnish Maternity Cohort did not find significant association between ovarian cancer and serum 25(OH)D<sub>3</sub> levels.

A concluding comment is that a high number, but by no means not all, observational, epidemiological, and pre-clinical studies suggest a protective anticancer action of vitamin D. The Cochrane review [66] warns for study bias due to low numbers of participants and selective groups of participants. A few aspects that need to be considered, as they may explain the not always consistent observations and can help to improve analyses and deepen our knowledge. First, what is sufficient and what is deficient? Criteria for sufficient and deficient are still related to classical end points in calcium and bone homeostasis. One can envisage that for cancer-related endpoints, different definitions of deficient and sufficient are needed. Second, associations are based on serum levels and then primarily on 25(OH)D levels. With new analytical tools and techniques, it is possible to produce a full vitamin D metabolic profile. These profiles can also be put into perspective of seasonal dynamics. In addition, the relationship between serum levels/serum metabolic profiles and target tissue or tumor levels/metabolic profiles should be taken into account [67]. Over the years, data have been accumulating on the relationship between obesity and vitamin D storage in adipose tissues on one hand and circulating levels of vitamin D on the other hand [68–71].

### **2.1.2 Genetic polymorphisms in the vitamin D system**

The majority of the genetic association studies have focused on variations in the VDR. Several polymorphisms have been identified in the VDR gene and studied in relation to various endpoints including osteoporosis and other diseases. Over the past 20–25 years, an increasing number of studies have been published focusing on the association of various polymorphisms in the VDR gene and different types of cancer. Overall, the size of these association studies is small and inadequate to control for confounding, consequently leading to a variety of inconclusive results. The cancer types investigated, a.o. prostate, breast, lung colorectal, skin, renal carcinoma, etc. are not

monogenetic disorders, and the impact of genetic variations will be small and dependent on interaction with polymorphisms in other genes and interaction with environmental factors. To reach robust conclusions on associations of polymorphisms with cancer incidence, progression, and/or treatment, large samples size studies are needed with uniform clinical endpoints and distinct description of phenotypic and environmental parameters. This will hopefully provide sufficiently powered studies for strong genotype–phenotype association analyses and gene–environment interactions as well as ethnic differences [72]. Combining genome-wide association studies in different cohort studies is an approach to follow, like the one on prostate cancer (BPC3) [73]. One of the conclusions by the authors is that more studies with larger numbers of participants are needed. An alternative intermediate step can be metaanalyses of the currently published smaller association studies. Recently, several attempts have been made in this direction [74–76] finding some associations but with a general conclusion that additional larger studies are needed. Publication bias is an important aspect to take into account with metaanalyses as studies showing an association are more likely published than those not showing an association.

Mendelian randomization (MR) is an increasingly popular approach to investigate the causal implications of gene variants and thereby to translate correlation to causation. At least 17 articles with MR in the title have been published from 2016 to 22 in the field of vitamin D and cancer. However, also for vitamin D and cancer, MR study sample size is important and challenging to identify a strong genetic marker and make these studies feasible. Vitamin D–cancer MR studies focused mainly on the relationship between genetic variants and circulating 25(OH)D levels and indirectly with cancer outcome (see, for example [77]). Following this, from the initial focus on VDR polymorphisms, an increasing number of studies include vitamin D metabolizing enzymes, including DHCR7, CYP2R1, CYP27B1, and CYP24A1 (e.g., Refs. [78–80]). But also here the aforementioned comments on sample size and phenotypic description are valid and important for robust conclusions. Further detailed discussion of associations, possible functional consequences of VDR, other gene polymorphisms, and impact of vitamin D levels are beyond the scope of this chapter but will be addressed in greater detail in Chapter 61.

## **2.2 Growth, development, and carcinogenesis**

In addition to the epidemiological studies and demonstration of VDR in cancer cells, since the early 1980s, there is also an increasing amount of cell

biological data supporting a role for vitamin D as an inhibitor of cancer growth [38,81–83]. Multiple studies have shown that at elevated concentrations ( $10^{-9}$ – $10^{-7}$  M),  $1,25(\text{OH})_2\text{D}_3$  inhibits the growth of tumor cells in vitro. It was demonstrated as early as 1981 that  $1,25(\text{OH})_2\text{D}_3$  inhibits the growth of malignant melanoma cells and stimulates the differentiation of immature mouse myeloid leukemia cells in culture [84–86].  $1,25(\text{OH})_2\text{D}_3$  also induces differentiation of normal bone marrow cells. Immature bone marrow cells of the monocyte–macrophage lineage are believed to be the precursors of osteoclasts, and  $1,25(\text{OH})_2\text{D}_3$  induces differentiation of immature myeloid cells toward monocytes–macrophages and also stimulates the activation and fusion of some macrophages. From these results, it has been postulated that  $1,25(\text{OH})_2\text{D}_3$  stimulates differentiation and fusion of osteoclast progenitors into osteoclasts [87–89]. These in vitro findings were followed by the in vivo observation that  $1,25(\text{OH})_2\text{D}_3$  prolongs the survival time of mice inoculated with myeloid leukemia cells [90]. As shown in Table 84.2, over the years,  $1,25(\text{OH})_2\text{D}_3$  has been shown to have beneficial effects in several other in vivo animal models of various types of cancers [91–113]. For more recent reviews for breast, colon, and prostate cancers, see Chapters 88, 89 and 91.

A relationship between vitamin D, the presence of VDR and carcinogenesis was shown for the skin. Absence of VDR increased the sensitivity for chemically induced tumorigenesis [114]. Vitamin D compounds protect against UV-induced DNA damage and photocarcinogenesis and reduce ROS/RNS to limit UV-induced suppression of immune response [115]. Moreover, in mice, the vitamin D analog EB1089 prevented  $\beta$ -catenin-induced trichofolliculomas, while low levels of VDR associated with the induction by  $\beta$ -catenin of infiltrative basal cell carcinomas [116]. The  $\beta$ -catenin as well as the Hedgehog signaling and the recently found lncRNA pathways underly the protective role of the VDR as a tumor suppressor in the skin [117,118]. In addition, regulation of *c-MYC* by the VDR may lie at the basis for cancer preventive actions [119]. In stroma from pancreatic tumors, the VDR is a master transcriptional regulator of the conversion to quiescent cells after calcitriol treatment, leading to reduced tumor volume and increase in survival compared with chemotherapy [7]. Also for prostate, colon, and liver, a protective role for vitamin D in carcinogenesis has been described [120–122]. A study with aging (up to 600 days) wild type and CYP27B1 knockout mice demonstrated that  $1,25(\text{OH})_2\text{D}_3$  deficiency leads to accelerated tumor initiation and growth. This is related to elevated oxidative stress, activating oncogenes, inactivation of tumor suppressor genes, and alteration of cellular senescence in tumor microenvironment [123]. An interesting area that

needs further exploration is the interplay between oral microbiota and oropharyngeal squamous cell carcinogens and the role of vitamin D herein [124]. A preclinical study reported vitamin D preventive effects on oral carcinogenesis, which depends on timing and duration of treatment implicating a limiting role for CYP24A1 [125].

An important aspect and limitation of the treatment of cancer with  $1,25(\text{OH})_2\text{D}_3$  to achieve growth inhibition are the relatively higher doses of  $1,25(\text{OH})_2\text{D}_3$  needed (confirming the in vitro data), which can cause the side effect of hypercalcemia (see Section 2.3). This has prompted the development of analogs of  $1,25(\text{OH})_2\text{D}_3$  in an attempt to dissociate the antiproliferative effect from the calcemic and bone metabolism effects. Although the precise mechanism for this dissociation of activities is not completely understood, at the moment several  $1,25(\text{OH})_2\text{D}_3$  analogs are available that seem to fulfill these criteria. In Table 84.3, the in vivo animal studies using  $1,25(\text{OH})_2\text{D}_3$  analogs on various cancer types are summarized [101,107,108,110–113,126–144].

## 2.3 Clinical studies

Only a limited set of clinical trials of vitamin D in cancer have been performed up to now. We will mention a few examples here. Further discussion on clinical trials can be found in the chapters on the specific malignancies that follow. Alfacalcidol ( $1\alpha$ -hydroxyvitamin  $\text{D}_3$ ;  $1\alpha$ -(OH) $\text{D}_3$ ), which is converted to  $1,25(\text{OH})_2\text{D}_3$  in vivo, caused a beneficial response in low-grade non-Hodgkin's lymphoma patients [145,146]. Also, in a study treating myelodysplasia patients with alfacalcidol, transient improvement in peripheral blood counts was seen; however, half of the patients developed hypercalcemia [147]. Another study reported a sustained hematological response in six myelodysplasia patients treated with high doses of alfacalcidol [148]. These patients were restricted in their dietary calcium intake; nevertheless, four patients developed hypercalcemia due to increased bone resorption. With respect to treatment of cutaneous T cell lymphoma with a combination of  $1,25(\text{OH})_2\text{D}_3$  and retinoids, contrasting results have been obtained. It has been suggested that the variability was due to differences in phenotype of the various lymphomas [149–151].

A study on early recurrent prostate cancer showed that daily treatment with  $1,25(\text{OH})_2\text{D}_3$  slowed the rise in prostate-specific antigen (PSA) [152]. Using a regime of once weekly treatment with very high-dose calcitriol was found to be safe but did not result in a significant reduction in PSA in prostate cancer cells [153]. Two studies were specifically designed to examine the route

**TABLE 84.2** In vivo effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1 $\alpha$ -(OH)D<sub>3</sub> in animal models of cancer.<sup>a</sup>

Tumor	Model	Effect	Refs
Adenocarcinoma	CAC-8 cells injected in nude mice	Reduction in tumor volume	[111]
Breast	NMU- and DMBA-induced breast cancer in rats Injection of fibroblasts from human breast tumors in nude mice and injection of treated mammospheres	Tumor suppression Reduced tumor volume	[97,100] [565]
Colon	Human colon cell line implanted into nude mice; DMH-induced colon cancer in rats; APCmin mice	Tumor suppression; reduction of the incidence of colon adenocarcinomas; decrease in polyp number and tumor load	[94,96,99,600]
Kaposi sarcoma	KS Y-1 cells implanted in nude mice	Tumor growth retardation	[109]
Leydig tumor	Leydig cell tumor implanted into rats	Tumor suppression	[101]
Liver tumor	Injection of liver carcinogen diethylnitrosamine in mice and low vitamin D diet	Increase in tumor growth	[436]
Lung	Implantation of Lewis lung carcinoma into mice	Reduction of the number of metastases (without suppression of primary tumor); tumor suppression; increased antitumor immunity	[91,105,466,601]
Melanoma	Human melanoma cells implanted into nude mice	Tumor suppression	[94]
Osteosarcoma	Human osteosarcoma cells implanted into nude mice	Tumor suppression	[102]
Prostate	Dunning MAT LyLu rat prostate model; LNCaP xenografts in nude mice; PAIII tumors in Lobund-Wistar rats	Reduction in lung metastasis; tumor suppression	[107,108,110,112,113]
Retinoblastoma	Retinoblastoma cell line implanted into nude mice; transgenic mice with retinoblastoma	Tumor suppression	[95,98]
Walker carcinoma	Walker carcinoma cells injected in rats	Tumor suppression	[104]
Skin	DMBA/TPA-induced skin tumors in mice Human squamous cell carcinoma cells (A431) injected in nude mice	Inhibition of tumor formation Tumor cell death	[92,93] [495]

<sup>a</sup>The dosage, duration of treatment, diet, and effects on serum/urinary calcium vary among the studies. DMBA, 7,12-dimethylbenz[a]anthracene; DMH, 1,2-dimethylhydrazine dihydrochloride; NMU, nitrosomethylurea; TPA, 12-O-tetradecanoylphorbol-13-acetate.

and schedule of administration and calcemic response in patients with advanced malignancies [154,155]. The set of trials using very high dose 1,25(OH)<sub>2</sub>D<sub>3</sub> plus taxotere in advanced prostate cancer is discussed in Chapter 91 on prostate cancer (Nonn & Campbell) and has recently been reviewed [156].

Clinical trials using vitamin D analogs have been initiated over the past years. However, these were

mostly limited clinical trials focusing on small groups of patients for whom regular treatment had failed. Only a relatively few studies have been published. The analog calcipotriol (Daivonex/Dovonex/MC903) has been used for topical treatment of advanced breast cancer; however, several of the patients still developed hypercalcemia [157]. Studies have been carried out in advanced breast cancer [158] and pancreatic cancer

**TABLE 84.3** In vivo effects 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs in animal models for cancer.

Analog	Model	Antitumor effect	Refs.
1,25(OH)D <sub>2</sub>	Retinoblastoma	Tumor suppression	[142]
1,25(OH)D <sub>5</sub>	Breast	Tumor suppression	[143]
CB966	Breast	Tumor suppression	[128]
CB1093	Prostate	Tumor suppression No effect on angiogenesis	[112]
DD-003	Colon	Tumor suppression	[134]
EB1089	Adenocarcinoma	Tumor suppression	[111]
EB1089	Breast	Tumor suppression	[128,131,139,492]
EB1089	Colon	Tumor suppression	[138]
EB1089	Hepatocellular carcinoma	Inhibition of tumor incidence	[602]
EB1089	Leydig cell tumor	Tumor suppression	[101]
EB1089	Prostate	Tumor suppression Reduction lung metastases No effect on angiogenesis	[108,110,112,113,140,141]
KH1060	Prostate	Tumor suppression	[113]
LG190119	Prostate	Tumor suppression	[110]
OCT	Breast	Tumor suppression	[127,132]
OCT	Breast	Tumor suppression	[129]
OCT	Breast	Tumor suppression	[132]
OCT	Colon	Decreased tumor incidence	[135]
MC903	Breast	Tumor suppression	[130]
Ro 23-7553	Prostate	Tumor suppression	[136]
Ro 23-7553	Leukemia	Increased survival	[126]
Ro 24-5531	Breast	Decreased tumor incidence	[133]
Ro 24-5531	Colon	Decreased tumor incidence	[137]
Ro-25-6760	Prostate	Tumor suppression	[107]
Ro-26-9114	Colon	Decrease in polyp number and tumor load	[600]
Ro-26-9114	Prostate	Tumor suppression	[113]

CB1093, 20-epi-22(S)-ethoxy-23yne-24a, 26a,27a-trihomo-1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; CB966, 24a,26a,27a-tri-homo-1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; DD-003, 22(S)-24-homo-26,26,26,27,27-hexafluoro-1 $\alpha$ ,22,25-trihydroxy-vitamin D<sub>3</sub>; EB1089, 22,24-diene-24a,26a,27a-trihomo-1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; MC903, 1,24-dihydroxy-22-ene-24-cyclopropyl-vitamin D<sub>3</sub>; OCT, 22-Oxacalciitrioloxacalciitriol; Ro 23-7553, 1,25dihydroxy-16-ene-23-yne-vitamin D<sub>3</sub>; Ro 24-5531, 1,25dihydroxy-16-ene-23-yne-26,27-hexafluorovitamin D<sub>3</sub>; Ro 26-9114, 1 $\alpha$ ,25-(OH)<sub>2</sub>-16-ene-19-nor-24-oxo-D<sub>3</sub>.

[159], but the clinical results were limited. In a single case of Kaposi sarcoma and topical application of calcipotriol, success in tumor regression was reported [109]. Also the impact of inhibition of CYP24A1 to enhance the anticancer activity of vitamin D has been studied, and a potentiation of the vitamin D effect was found as has been shown previously in vitro [160]. Data on clinical studies with vitamin D and vitamin D analogs are reviewed by Vijayakumar [161,162], Feldman [38], Giammanco [163], and Scaranti [164].

More well-designed, sufficiently powered randomized double-blind placebo-controlled trials with primary cancer-related endpoints are necessary to overcome some unsolved issues in previous studies involving younger participants, men/women, and taking into account individual vitamin D status, duration of treatment, dosage levels, and longer follow-up of all participants. Metaanalysis of randomized controlled trials is an alternative approach like the recent one for breast cancer [165]. This analysis led to the conclusion



that there is insufficient evidence to support efficacy of vitamin D supplementation but the cautionary comments on metaanalysis mentioned in the [Section 2.1.2](#) also hold for the clinical trials.

Together, limitations in study design may be part of the explanation of the unambiguous clinical study results, but it can also reflect that vitamin D on its own may not be a strong and primary anticancer drug.

## 2.4 Angiogenesis and metastasis

For the tumor suppressive activity of vitamin D<sub>3</sub> compounds *in vivo*, besides growth inhibition and differentiation, two additional aspects contribute to potential benefits including effects on (1) angiogenesis and (2) invasion and metastasis. First, we will discuss vitamin D and angiogenesis. Angiogenesis is an essential requirement for the growth of solid tumors. Compounds that inhibit angiogenesis might therefore contribute to anti-tumor therapy. Antiangiogenic drugs may lead to inhibition of tumor progression, stabilization of tumor growth, tumor regression, and prevention of metastasis. Antiangiogenic effects may play a role in the tumor suppressive activity of vitamin D<sub>3</sub> compounds [166]. The effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on angiogenesis may be due to inhibition of tumor cell proliferation, resulting in fewer angiogenic cells. However, inhibition of angiogenesis could also be observed when the tumor cells were treated *in vitro* with 1,25(OH)<sub>2</sub>D<sub>3</sub> and, after cell washing, were injected into mice [167]. Under these conditions, both control and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mice were injected with similar numbers of cells. Therefore, these data indicate that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the release of angiogenic factors (vascular endothelium growth factor, transforming growth factor- $\alpha$ , basic fibroblast growth factor, epidermal growth factor, etc.) or stimulates antiangiogenic factors. 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment caused a reduction in the angiogenic signaling molecule, angiopoietin-2 in squamous cell carcinoma, and radiation-induced fibrosarcoma-1 cells [168]. 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited both VEGF production and HIF1 $\alpha$  transcriptional activity in cancer cells of various origins under both normoxic (20% oxygen) and hypoxic (1% oxygen) conditions [169]. 1,25(OH)<sub>2</sub>D<sub>3</sub> also reduced HIF1 $\alpha$  and VEGF in an *in vivo* prostate tumor model, which was accompanied by an abnormal tumor angiogenesis. This was only observed in wild-type but not in VDR knockout tumors [170]. In retinoblastomas in mice, 1,25(OH)<sub>2</sub>D<sub>3</sub> has also been shown to reduce angiogenesis [171].

A study by Oades et al. [112] however, showed that the 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs EB1089 and CB1093 inhibited tumor growth in two prostate animal models but did not inhibit angiogenesis in a rat aorta assay. Whether this implicates that vitamin D affects angiogenesis in

a tumor situation and not in a nonmalignant condition is not clear. This may resemble the effects of endostatin, which inhibits pathological but not normal vascularization [172,173]. In support of this possibility is the finding that 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs EB1089, Ro-25-6760, and ILX23-7553 potently inhibit growth of endothelial cells derived from tumors but are less potent against normal aortic or yolk sac endothelial cells [168]. In SW480-ADH colon cancer cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> has a complex regulatory effect on the angiogenic phenotype: it increases the expression of VEGF and TSP-1 and regulates Id genes, but not that of PDGF-B, through the activation of their respective promoters [174]. An interesting observation is that also deglycosylated vitamin D-binding protein (DBP-maf) has been reported to inhibit angiogenesis [175,176] and to inhibit growth of pancreatic tumors in nude mice [176]. Whether 1,25(OH)<sub>2</sub>D<sub>3</sub> may interfere with DBP-maf in tumor growth inhibition and antiangiogenesis remains to be established. Interaction with another factor, interleukin-12, in the inhibition of angiogenesis has been reported [177]. The data so far show that interference with angiogenesis is a general effect of vitamin D, via effects either direct on endothelial cells or indirectly via effects on the cancer cells.

A mechanism of antitumor activity to be discussed, and one related to angiogenesis, is invasion and metastasis. Metastasis is the primary cause of the fatal outcome of cancer diseases. A study by Mork Hansen et al. [178] indicated that 1,25(OH)<sub>2</sub>D<sub>3</sub> may be effective in reducing the invasiveness of breast cancer cells. They showed that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the invasion and migration of a metastatic human breast cancer cell line (MDA-MB-231) using the Boyden chamber invasion assay. In support of this, it was shown that 1,25(OH)<sub>2</sub>D<sub>3</sub>, and the analogs KH1060, EB1089, and CB1093, all inhibited secretion of tissue-type and urokinase plasminogen activator and increased plasminogen activator inhibitor 1 (PAI-1) in the MDA-MB-231 metastatic breast cancer cell line [179].

In line with decreasing the capability of breast cancer cells to metastasize, 1,25(OH)<sub>2</sub>D<sub>3</sub> also inhibited the epithelial–mesenchymal transition, an important step in metastatic behavior [180]. Current understanding of the role of vitamin D in the epithelial–mesenchymal transition is reviewed by Munoz and coworkers [181–183]. Since then, multiple new studies have been published further demonstrating the vitamin D inhibition of epithelial–mesenchymal transition in a variety of cancer types. Among others, Horas et al. [184] demonstrated a role for the VDR in preventing epithelial–mesenchymal transition. Noncoding RNAs are important regulators in cellular (patho)physiology. The mir1204 stimulates tumor growth and targets VDR. Silencing of mir1204 increased expression of

VDR mRNA. VDR and mir1204 oppositely regulate cancer cell proliferation, metastasis, and epithelial–mesenchymal transition [185].

The vitamin D analog EB1089 also prevented skeletal metastasis in vivo and prolonged survival time in nude mice transplanted with human breast cancer cells [186]. Interestingly, it was shown that vitamin D deficiency promotes the growth of human breast cancer cells in the bones of nude mice [187]. This metastatic behavior in vitamin D deficiency is tumor autonomous and involves abrogation of the expression of the tumor progression gene Id1 [188].

Interestingly, the “prohormone” 25(OH)D could delay neoplasia, tumor growth, and metastasis in a non-immunodeficient MMTV-PyMT mouse model of metastatic breast cancer [189].

Vitamin D also inhibited the invasive ability of human prostate cancer cell lines, LNCaP, PC-3, and DU 145. 1,25(OH)<sub>2</sub>D<sub>3</sub> decreased MMP-9 and cathepsins, but not plasminogen activities, while it increased the activity of tissue inhibitors of metalloproteinase-1 (TIMP-1) and cathepsin inhibitors [190]. An intriguing observation is the interplay between VDR and TRPV5 in metastasis of renal cell carcinoma. TRPV5 plays a crucial role in renal calcium reabsorption, and its expression is directly upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [191,192]. However, in renal carcinoma cell lines, VDR knockdown led to increased TRPV5 expression accompanied by an increase in proliferation and migration while additional knockdown of TRPV5 reversed these effects on the tumor cells [193].

In an in vivo study, it was shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces the metastasis to the lung of subcutaneously implanted Lewis lung carcinoma cells [105]. In two animal models of prostate cancer, 1,25(OH)<sub>2</sub>D<sub>3</sub> and the analogs EB1089 and RO25-6760 inhibited lung metastases [107,108]. In these models, the tumors were implanted subcutaneously, and therefore, in contrast to the model of direct tumor cell injection in the left ventricle [194], no bone metastases occurred. In pancreatic cancer, the vitamin D analog MART-10 as well as 1,25(OH)<sub>2</sub>D<sub>3</sub> repressed migration and invasion of tumor cells via blocking the epithelial–mesenchymal transition [195]. MART-10 was also reported to repress metastases of head and neck squamous carcinoma cells and migration of breast cancer cells [196,197]. MART-10 inhibited VEGF-A-induced breast cancer and neuroendocrine tumor cell migration, suggestive of the interplay in control of angiogenesis and metastasis [198].

A fact to be considered in relation to metastasis is that bone is the most frequent site of metastasis of advanced breast and prostate cancer. There are some indications from clinical studies that bone metastases develop preferentially in areas with high bone turnover [199,200]. In contrast, agents that inhibit bone resorption such as

bisphosphonates and Denosumab have been reported to reduce the incidence of skeletal metastasis and improve survival [201–204]. Promising are also studies that focus on bone anabolic therapies [205]. Akech et al. [206] showed that Runx2 is a key regulator of events associated with prostate and breast cancer metastatic bone disease. Runx2 is intimately involved in vitamin D actions in osteoblast development [207]. As 1,25(OH)<sub>2</sub>D<sub>3</sub> may stimulate bone turnover, treatment of cancer with 1,25(OH)<sub>2</sub>D<sub>3</sub> might theoretically increase the risk of skeletal metastases. 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits breast cancer cell migration toward a bone environment in an in vitro bone metastasis model [208]. However, this in vitro model only focuses on the direct 1,25(OH)<sub>2</sub>D<sub>3</sub> effects on cancer cells but lacks any effect of vitamin D on bone turnover. Further studies in in vitro models that more closely resemble the in vivo situation and/or in vivo studies are needed to address this aspect of 1,25(OH)<sub>2</sub>D<sub>3</sub> therapy. Considering the use of vitamin D<sub>3</sub> analogs with reduced calcemic activity or treatment with vitamin D<sub>3</sub> in combination with other compounds to reduce bone turnover may be helpful (see Section 4). The versatile aspects of endocrine interplay (including vitamin D) in the cross-talk between bone cells and metastatic cancer cells are reviewed by Hofbauer et al. [209].

1,25(OH)<sub>2</sub>D<sub>3</sub> decreased androgen-stimulated progression of prostate cancer, but prolonged treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> increased metastatic behavior in a model of transgenic adenocarcinoma of mouse prostate (TRAMP). This shows the need for further mechanistic studies to elucidate these both antineoplastic as well as prometastatic effects of vitamin D in prostate cancer [210]. Increased metastasis has also been observed in a 4T1 breast cancer mouse model after 1,25(OH)<sub>2</sub>D<sub>3</sub> or the analogs PRI-2191 and PRI-2205 treatment [211,212]. The authors study the effects on the immune cells in the tumor microenvironment and explain the increased metastasis by immunosuppressive effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs [212]. These data further stress the significance to focus on the complete composition of the tumor including cancer, stromal, and immune cells to fully understand and eventually predict a beneficial effect of vitamin D in cancer treatment.

## 2.5 Parathyroid hormone–related peptide

1,25(OH)<sub>2</sub>D<sub>3</sub> and parathyroid hormone (PTH) mutually regulate synthesis and secretion of one another. Production and secretion of PTH are inhibited by 1,25(OH)<sub>2</sub>D<sub>3</sub> via a transcriptional effect, and a vitamin D responsive element (VDRE) in the promoter of the PTH gene has been identified [213,214]. Parathyroid hormone–related peptide (PTHrP) was initially isolated

from several carcinomas and is responsible for the syndrome of humoral hypercalcemia of malignancy [215,216]. Although originally identified in carcinomas, PTHrP has also been identified in normal cells. As will be discussed now, vitamin D effects to inhibit PTH and PTHrP may have a role in its anticancer actions and in reducing metastases to bone [217,218].

In normal human mammary epithelial cells,  $1,25(\text{OH})_2\text{D}_3$  did not affect basal but inhibited growth factor–stimulated PTHrP expression via an effect on transcription [219]. In normal keratinocytes,  $1,25(\text{OH})_2\text{D}_3$  had no effect on PTHrP secretion in basal culture conditions [220] but did inhibit growth factor–stimulated PTHrP production as well [221]. Likewise,  $1,25(\text{OH})_2\text{D}_3$  as well as the analogs 22-oxacalcitriol and MC903 inhibited PTHrP secretion in immortalized human keratinocytes (HPK1A), but this inhibition was less in the more malignant ras-transfected clone HPK1A-ras [222,223].  $1,25(\text{OH})_2\text{D}_3$  and the analogs EB1089 and 22-oxacalcitriol inhibited the PTHrP gene transcription in and release from the squamous cancer cell line NCI H520 [224].

In the human T cell lymphotropic virus type I (HTLV-I)–transfected T cell line MT-2,  $1,25(\text{OH})_2\text{D}_3$  and 22-oxacalcitriol inhibited PTHrP gene expression and PTHrP secretion [225] and in rat H-500 Leydig tumor cells [226]; and  $1,25(\text{OH})_2\text{D}_3$  inhibited PTHrP secretion by PC-3 prostate cancer cells. However, another study demonstrated a prostate cancer–specific or cell-specific effect. Vitamin D and the analog EB1089 inhibited the PTHrP expression via a negative VDRE in LNCaP but not in PC3 prostate cancer cells [227,228]. It was suggested that this might play a role in the growth inhibition by vitamin D as PTHrP stimulates prostate cancer growth, tumor invasion, and metastasis [229–231]. In vivo observations comparable with these in vitro observations have also been made. When H-500 Leydig tumor cells were implanted in Fisher rats, treatment with  $1,25(\text{OH})_2\text{D}_3$  and the analog EB1089 resulted in reduced levels of tumor PTHrP mRNA and PTHrP serum levels [101]. EB1089 also reduced serum levels of PTHrP in nude mice implanted with squamous cancer cells [232].

In Fisher rats implanted with the Walker carcinoma,  $1,25(\text{OH})_2\text{D}_3$  caused a decrease in serum PTHrP, but the ratio of PTHrP levels and tumor weight was similar in rats receiving vehicle or  $1,25(\text{OH})_2\text{D}_3$ . The data point to an indirect effect on PTHrP via growth inhibition. However, the PTHrP mRNA levels appeared to be decreased by  $1,25(\text{OH})_2\text{D}_3$  [104]. In nude mice bearing the FA-6 cell line of a pancreas carcinoma lymph node metastasis, 22-oxacalcitriol inhibits PTHrP gene expression, which is related to inhibition of tumor-induced hypercalcemia [233]. Together, the overall picture that emerges from these studies is that an important

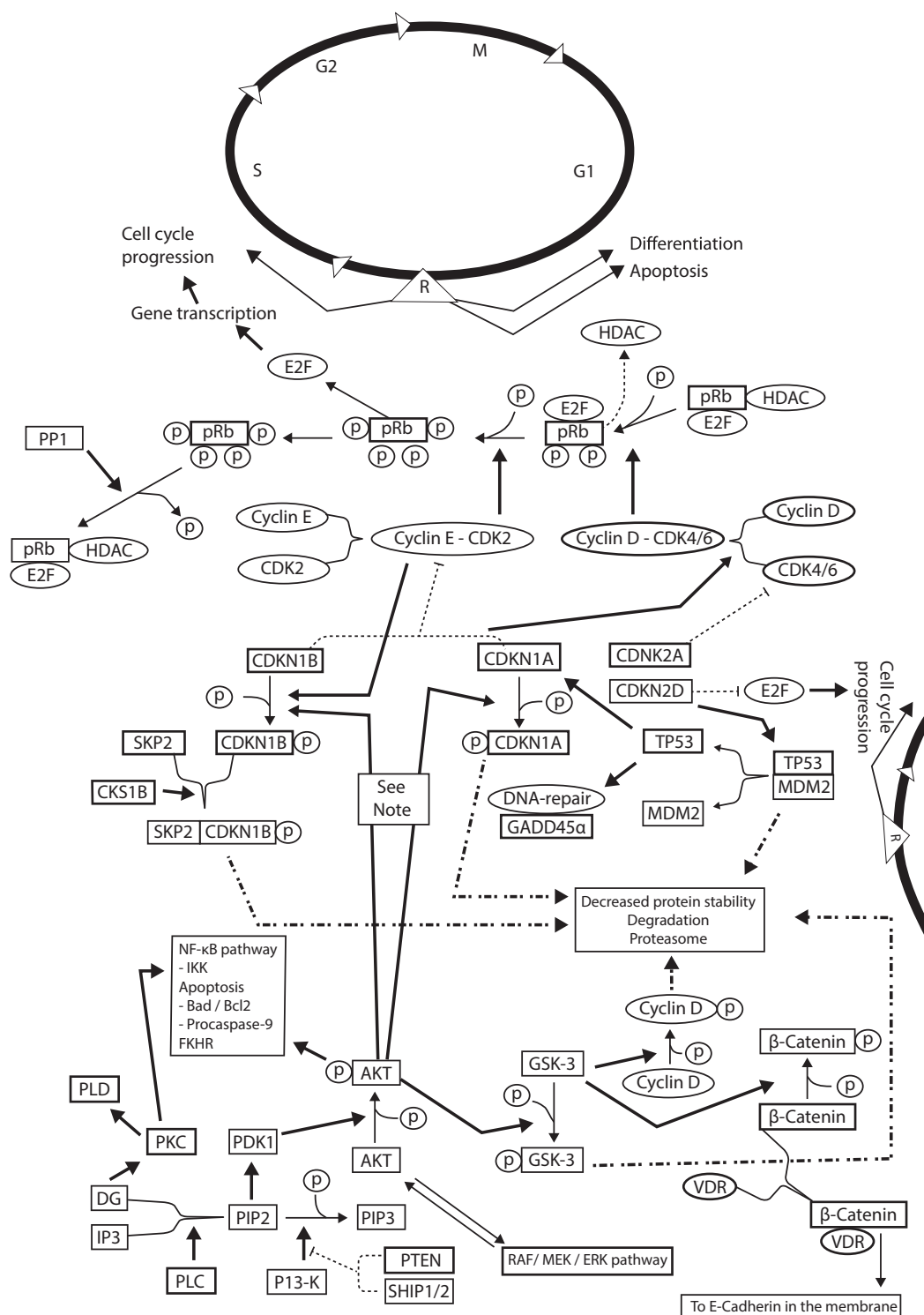
additional anticancer effect of vitamin D and analogs could be the inhibition of the humoral hypercalcemia of malignancy. However, also studies emerged reporting that PTHrP and  $1,25(\text{OH})_2\text{D}_3$  are both causing tumoral hypercalcemia. PTHrP and  $1,25(\text{OH})_2\text{D}_3$  were shown to be cosecreted in a non-Hodgkin's lymphoma patient and both together with hypercalcemia normalized after chemotherapy [234].

In contrast to these inhibitory effects in human tumor cells and tumor models, a stimulatory effect of  $1,25(\text{OH})_2\text{D}_3$  and EB1089 on PTHrP gene transcription and PTHrP production by a canine oral squamous carcinoma cell line (Sec 2/88) has been observed [235,236]. Also in vivo with the canine adenocarcinoma CAC-8 in nude mice, stimulation of PTHrP by  $1,25(\text{OH})_2\text{D}_3$  and EB1089 was observed [236]. These data indicate that the effect of vitamin D and analogs on canine tumors differs from that on human tumors.

### 3. Vitamin D effects on tumor cells

#### 3.1 Molecular action: (proto)-oncogenes and tumor suppressor genes

Oncogenes and tumor suppressor genes generally are involved in control of the cell cycle and apoptosis. One of the most widely studied oncogenes in relation to vitamin D is *c-MYC*. *c-MYC* suppresses expression of cell cycle/growth arrest genes *gas1*, *CDKN2B* (p15), *CDKN1A* (p21), *CDKN1B* (p27), and *gadd34*, *gadd45*, and *gadd153* [237] and has been postulated to play an early role in the following cascade of events in  $G_1$ : cyclins activate cyclin-dependent kinases (CDKs), which in turn can phosphorylate the retinoblastoma tumor suppressor gene product (p110<sup>RB</sup>), resulting in transition from  $G_1$  to S phase (see Fig. 84.1). In several cancer cell types,  $1,25(\text{OH})_2\text{D}_3$  has been reported to decrease *c-MYC* oncogene expression [238]. Analysis of HL-60 sublines showed a relation between reduction of *c-MYC* expression and inhibition of proliferation [239]. Similar observations were made for neuroblastoma cells treated with  $1,25(\text{OH})_2\text{D}_3$ , EB1089, and KH1060 [240]. The mechanism of *c-MYC* inhibition appears to be both direct, by inducing the binding of proteins to an intron element and the involvement of HOXB4 [241,242], and at least in colon cancer cells also indirect via the inhibition of the transcriptional activity of  $\beta$ -catenin and T cell factor (TCF) complexes [243]. In earlier studies, we did not observe a  $1,25(\text{OH})_2\text{D}_3$ -induced change in *c-myc* expression in MCF-7 and ZR-75.1 breast cancer cells while they were both growth inhibited [244], and a similar observation has been made for the colon adenocarcinoma CaCo-2 cell line [245]. Nontransformed embryonic fibroblasts are growth inhibited by



**FIGURE 84.1** Schematic representation summarizing the intracellular pathways and signaling pathways involved regulation of the cell cycle shown to be regulated by  $1,25(\text{OH})_2\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  analogs in regulating cell proliferation. Targets shown to be affected by  $1,25(\text{OH})_2\text{D}_3$  and/or its analogs are indicated in the bold boxes and ovals. Bold arrows and fine dotted lines indicate stimulation and inhibition, respectively. Coarse dotted lines indicate processing to the proteasome. p indicates phosphorylation. The effects on these cellular targets are not demonstrated in all types of cancer cells, but this diagram is aimed to give an overview of demonstrated targets and potential targets. Note: Dependent on the site of phosphorylation proteins can either be destabilized or degraded or be stabilized and activated. For example, phosphorylation of CDKN1A at T145 by AKT leads to degradation, while phosphorylation of S146 by AKT leads to increased stability. *AKT* (*PKB*), Protein kinase B; *Bad*, BCL2-antagonist of cell death; *Bcl2*, B cell leukemia/lymphoma 2; *Cdk*, Cyclin-dependent kinase; *CKS-1*, Cyclin kinase subunit 1; *DG*, Diacylglycerol; *E2F*, transcription factor; *ERK*, Extracellular signal-regulated kinase; *FKHR* (*AFX/FOX*), Forkhead family of



1,25(OH)<sub>2</sub>D<sub>3</sub>, whereas *c-myc* is not changed or is even increased [246,247]. In the MG-63 osteosarcoma cell line, 1,25(OH)<sub>2</sub>D<sub>3</sub> has been shown to enhance *c-MYC* expression [248], whereas we observed growth inhibition by 1,25(OH)<sub>2</sub>D<sub>3</sub> [249]. Likewise, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits proliferation and increases *c-MYC* expression in fibroblasts from psoriatic patients [250].

In a recent study, inhibition of *c-myc* was implicated as playing a major role in the ability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to inhibit prostate cancer proliferation [251]. As an underlying mechanism, 1,25(OH)<sub>2</sub>D<sub>3</sub> and the VDR regulate the functional balance of *c-MYC* and its repressor MAD1/MXD1, to suppress *c-MYC* function [119].

Collectively, these data show that regulation of *c-myc* expression may be part of growth inhibition by vitamin D but that this is not generally applicable to all cells. 1,25(OH)<sub>2</sub>D<sub>3</sub> has also been reported to regulate expression of other oncogenes [252–254]; however, these data are rather limited. Nevertheless, it is clear that 1,25(OH)<sub>2</sub>D<sub>3</sub> has effects on the expression of various proto-oncogenes. The data so far are not conclusive with respect to which genes are crucial in the growth inhibitory action of 1,25(OH)<sub>2</sub>D<sub>3</sub>. This can be attributed to the fact that these (proto)oncogenes encode for transcription factors, growth factor receptors or components, or intracellular signaling cascades. The effects of these genes may differ between cells dependent on the presence or absence of additional cell type–specific conditions. Therefore, their postulated role is often complex. For example, increased *c-MYC* expression can be related to induction of apoptosis but also to stimulation of cell cycle progression. Interestingly, in oncogene-induced senescence, functional relationships were revealed between Ras, the vitamin D/VDR axis, and DNA repair factors [255].

In contrast to the oncogenes, the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on tumor suppressor genes like the retinoblastoma gene is much clearer. This may be related to the fact that, in contrast to oncogenes, retinoblastoma and TP53 take well-defined positions in the control of cell cycle and DNA repair (see Fig. 84.1). The p110<sup>RB</sup> retinoblastoma gene product can either be phosphorylated or dephosphorylated. In the phosphorylated form, it can activate several transcription factors and causes transition to S phase and DNA synthesis [256]. In human chronic myelogenous leukemia cells [257], breast cancer cells [258], and HL-60 cells [259,260], 1,25(OH)<sub>2</sub>D<sub>3</sub> caused a dephosphorylation of p110<sup>RB</sup>, which is related to growth

inhibition and cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> and in one study also in G<sub>2</sub> [260]. In the leukemic cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> also caused a reduction in the cellular level of p110<sup>RB</sup> [257,259]. In nontransformed keratinocytes, 1,25(OH)<sub>2</sub>D<sub>3</sub> induced dephosphorylation of p110<sup>RB</sup> as well [261]. The other major tumor suppressor gene is TP53 (*p53*). For leukemic U937 cells, it was reported that presence of TP53 is important for 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation [262]. In rat glioma cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> induces expression of TP53 [263]. However, 1,25(OH)<sub>2</sub>D<sub>3</sub> can inhibit cell growth and induce differentiation in cancer cells with defective TP53 [264], and also TP53-independent induction of apoptosis by EB1089 has been demonstrated [265]. These latter observations might be explained by the fact that vitamin D also interferes at levels in the cascade of cell cycle control downstream of TP53 (see Fig. 84.1). Recently, novel interesting data were added to the story of TP53 and 1,25(OH)<sub>2</sub>D<sub>3</sub> [266]. It was shown that a mutant TP53, often present in tumors, physically and functionally interacts with VDR. Mutant TP53 is recruited to vitamin D target genes and can stimulate gene expression and relieve suppression of other genes. Mutant TP53 increases nuclear accumulation of VDR and transforms vitamin D into an antiapoptotic agent [266]. An interesting unique relationship between tumor suppressor genes and vitamin D has recently been shown for the Wilms' tumor suppressor gene WT1. This zinc finger–containing transcription factor induces transcription of the VDR gene [267].

Several interesting additional genes, interactions and vitamin D targets in cancer treatment should be mentioned. It has been demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> can trigger NF-κB activity through PI3K/Akt pathways [268,269], and also, treatment of NB4 leukemic cells with vitamin D causes a rapid phosphorylation of IκBα [270]. Contrary to these observations, vitamin D has been shown to inhibit NF-κB activity by increasing IκBα expression in different cell lines [271–273]. Sun et al. [274], using mouse embryonic fibroblasts derived from *Vdr*–/– mice, demonstrated that VDR plays an inhibitory role in NF-κB activation by regulating IκBα levels and VDR-RELA (p65) interaction. This role for VDR was supported by a recent study that also demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits transcriptional activity of NF-κB in breast cancer cells via histone deacetylase (HDAC3 and SMRT)–mediated p65 transrepression [275]. Kovalenko et al. [276] showed direct transcriptional

transcription factors; GSK-3, Glycogen synthase kinase-3; HDAC, Histone deacetylase; IKK, I-κB kinase; IP3, inositol 1,4,5-trisphosphate; Mdm2, Mouse double minute 2; MEK, Raf-1-MAPK/ERK kinase; PDK1, Phosphatidylinositol-dependent kinase 1; PI3-K, Phosphatidylinositol 3 kinase; PIP2, Phosphatidylinositol (4,5)-phosphate; PIP3, Phosphatidylinositol (3,4,5) phosphate; PKC, Protein kinase C; PLC, phospholipase C; PLD, Phospholipase D; PPI1, Protein phosphatase 1-like protein; pRB, Retinoblastoma protein; PTEN, Phosphatase and tensin homolog; SHIP 1 and 2, Src homology 2 (SH2) containing phosphatases 1 and 2; SKP2, Ubiquitin ligase; DR, Vitamin D receptor.

regulation by  $1,25(\text{OH})_2\text{D}_3$  of NF- $\kappa\text{B}$  in RWPE1 immortalized but nontumorigenic prostate cells. Fekrmandi et al. [277] found that  $1,25(\text{OH})_2\text{D}_3$  suppressed NF- $\kappa\text{B}$  function by enhancing the turnover of the FBW7-dependent subunit.  $1,25(\text{OH})_2\text{D}_3$  also indirectly inhibits NF- $\kappa\text{B}$  by directly stimulating expression of IGFBP-3, an inhibitor of NF- $\kappa\text{B}$  [278].

Interestingly, in relation to NF- $\kappa\text{B}$  regulation, as early as 1994, Chen and DeLuca isolated and characterized a vitamin D-induced gene in HL-60 cells [279]. The encoded protein, named vitamin D-upregulated protein-1 (VDUP1), is a thioredoxin-binding protein-2 [280]. Thioredoxin has several roles in processes such as proliferation or apoptosis. It also promotes DNA binding of transcription factors such as NF- $\kappa\text{B}$ , AP-1, TP53, and PEBP2. In addition, overexpression of thioredoxin suppresses the degradation of I $\kappa\text{B}$  and the transactivation of NF- $\kappa\text{B}$ , whereas overexpression of nuclear-targeted thioredoxin exhibits enhancement of NF- $\kappa\text{B}$ -dependent transactivation [281]. However, it is in only more recent studies that a coupling between VDUP1 and cancer has been made. The expression of VDUP1 was found to correlate with malignant status of colorectal and gastric cancers [282]. 5-Fluorouracil, which is widely used for treatment of colon cancer, induces VDUP1 expression in the SW620 colon cancer cell line [283]. In smooth muscle cells and cardiomyocytes, VDUP1 inhibits proliferation and is involved in induction of apoptosis [284,285]. A relation with vitamin D effects on cancer is made by two recent studies showing induction of VDUP1 by  $1,25(\text{OH})_2\text{D}_3$  in tumor cells and that VDUP1 induces cell cycle arrest [286,287]. Moreover, interaction with histone deacetylase (HDAC; see Fig. 84.1) and promyelocytic leukemia zinc finger (PLZF) was demonstrated. Interestingly and further complicating the story, PLZF inhibits  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation of U937 leukemic cells by binding to the VDR and inhibiting gene transcription [288,289]. Interestingly, a new related gene, DRH1, was cloned, and its expression was found to be strongly reduced in hepatocellular carcinoma tissue compared with normal liver [290]. DRH1 is 41% homologous with VDUP1. Whether this points to a new family of cancer genes remains to be established, but it certainly opens new venues for intervening in cancer cell growth.

Important in the regulation of gene expression is the involvement of noncoding RNAs, including microRNAs (miRNAs), long non-coding (lnc) RNAs, and small nucleolar (sno) RNAs [291]. These small endogenous RNAs target mRNAs and cause translational repression or degradation [292], thereby affecting a multitude of cellular functions including all processes identified to be vitamin D targets in cancer biology. In gastric cancer cells, it was found that miR-145 is induced by  $1,25(\text{OH})_2\text{D}_3$  and mediates antiproliferative effects on

gene regulation by vitamin D, with a direct target transcription factor E2F3 [293]. Also in other cancers, vitamin D regulates miRNA expression, which opens new routes for therapeutic targeting [294,295]. Several studies have shown that VDR itself is a target for miRNAs: miR-125b repressed endogenous levels of VDR in MCF-7 cells. Since miR-125b is downregulated in cancer, this may result in upregulation of the VDR and positively influence the antitumor effects of vitamin D [296]. Also, miR-1204 and miR-214 downregulate VDR expression in breast cancer cell lines [185,297]. The regulation of miRNAs by vitamin D in cancer model systems and impact on  $1,25(\text{OH})_2\text{D}_3$  signaling is reviewed by Ma et al. [298]. Developments in the noncoding RNA field are going fast. High-throughput techniques such as qPCR arrays and whole-genome sequencing will provide a broad inventory of vitamin D regulation of noncoding RNAs [297,299]. Also, the possibilities that noncoding RNAs packaged in exosomes can (1) signal between cells and (2) may serve as diagnostic markers are important innovations [300–304]. These developments warrant an updated review on noncoding RNAs, vitamin D, and cancer. Recently, also circular (circ) RNAs have gained a growing interest due to their broad expression and potential role in cancer [305]. The circular RNA hsa\_circ\_0060927 is generated from the CYP24A1 pre-mRNA and found to be expressed in colorectal cancer tissues and to be induced by  $1,25(\text{OH})_2\text{D}_3$  [306]. This latter might not be that surprising as the CYP24A1 gene is one of the most sensitive  $1,25(\text{OH})_2\text{D}_3$  genes. However, it may add a layer of complexity in the relationship between CYP24A1 and  $1,25(\text{OH})_2\text{D}_3$  in the regulation of cancer cells once we know the function of hsa\_circ\_0060927. The role of noncoding RNAs in vitamin D signaling and function is discussed in greater detail in Chapter 14.

Several alternate therapeutic targets for vitamin D anticancer activity can be mentioned here that are discussed in more detail in the following various chapters on specific cancers. Vitamin D regulates enzymes involved in estrogen and androgen synthesis and metabolism since these pathways drive the growth of breast and prostate cancer, respectively [307–311]. Vitamin D downregulates the expression of estrogen receptor (ER) $\alpha$  (ESR1). Two negative VDREs in the ER promoter act together in inhibiting ESR1 expression by  $1,25(\text{OH})_2\text{D}_3$  [312].

Telomerase activity provides a mechanism for unlimited cell division. In HL-60 cells,  $1,25(\text{OH})_2\text{D}_3$  inhibits telomerase activity, [313] and higher circulating vitamin D levels are associated to telomere length in leukocytes [314]. Additionally, whether the homeobox genes will prove to be a major target for vitamin D action in cancer remains to be elucidated, but in a differential expression screen in the human U937 leukemic cells, the HoxA10

gene was shown to be regulated by  $1,25(\text{OH})_2\text{D}_3$  [315]. A link with miRNA was made in gastric cancer. MiRNA-99b-3p is a direct target of vitamin D and reduces HoxD3 expression. Vitamin D also reduces HoxD3 expression suggestive for a link, but no definitive proof was provided that this vitamin D effect relies on induction of miRNA-99b-3p [316]. It was further suggested that the inhibitory effects on prostate cancer cell growth by vitamin D were related to the ability of  $1,25(\text{OH})_2\text{D}_3$  to modulate assembly of CX32 (GJB1) proteins into gap junctions, a way of cell–cell communication that is important in cell growth and differentiation [317]. For effects on other cell–cell junctions, see Section 3.4 on Differentiation.

A final but very important area is the antiinflammatory activity of vitamin D. Inflammation and carcinogenesis are intimately related, and immune cells in the tumor or vicinity of the tumor are critical determinants in tumor progression/regression. The currently most successful and promising cancer therapy is based on genetically engineered immune cells; chimeric antigen receptor (CAR)-T cells [318]. Yet, no data are available on vitamin D and CAR-T cells, but a broad range of effects on immune cells have been described and will be discussed in detail in other chapters. Vitamin D inhibits many proinflammatory pathways perhaps contributing to its chemoprevention as well as its therapeutic activity [278]. Stromal–epithelial cross-talk is important in the effects of  $1,25(\text{OH})_2\text{D}_3$  on the inflammatory process, as was shown in prostate cancer [319]. Vice versa, proinflammatory cytokines such as TNF $\alpha$  and IL-6 can decrease the expression of CYP27B1 in colon cancer, impairing activation of vitamin D, so limiting its antiinflammatory action again [320] and inflammation can induce CYP24A1 translation via an internal ribosome entry site [321].

Maybe not directly considered as a classical oncogenes, an oncogenic role for CYP24A1 is suggested by data showing amplification of the *CYP24A1* locus on chromosome 20q13.2 [322], and increased copy number causing overexpression in colorectal cancer [323]. CYP24A1A1 is mentioned as a new prognostic biomarker for colorectal cancer patients [324,325]. It should be noted that *CYP24A1* is located in a region at 20q13 that is found amplified in breast and ovarian cancers and that harbors about 300 other genes [326,327].

Several studies applied whole-genome profiling of  $1,25(\text{OH})_2\text{D}_3$ -treated cancer cells and patient explants. These large-scale genome-wide expression analyses will identify vitamin D–regulated genes and noncoding RNAs. These additional findings will add to the unraveling and further understanding of the mechanism of vitamin D control of cancer cell proliferation [328–330]. An RNA profiling study revealed 523 genes that were differentially expressed in breast cancer tissue

after vitamin D treatment (compared with 127 genes in normal breast tissue). These genes were mainly involved in processes such as cellular adhesion, metabolic pathways, and tumor suppressor–like pathways. Increased expression of three of these genes, *CLMN*, *SERPINB1*, and *KLK6*, is associated to prolonged relapse [331]. Tumor heterogeneity may be at the basis of the observation that in a comparative analysis of various breast cancer cells and tumor specimens, only four genes, *CYP24A1*, *CLMN*, *EFTUD1*, and *SERPINB1* were found to be upregulated in all samples. These four and *KLK6* were confirmed to be regulated by  $1,25(\text{OH})_2\text{D}_3$  in breast cancer cell lines and clinical specimens [18,332]. For sure, these whole genome-wide approaches together with single-cell and spatiotemporal expression profiling will provide a myriad of interesting molecular data. To optimally employ these data, a precise and robust description of and linkage to phenotypic/clinical data is critical.

### 3.2 Cell cycle and DNA repair

It has now been well established that vitamin D inhibits growth of cells by interfering with the cell cycle (see Chapter 86). In a randomized clinical trial, an inverse relation of vitamin D metabolite levels and Ki67 intensity (proliferative activity) in prostate cancer tissue was found after vitamin D treatment [333]. Both in breast cancer [334] and in colon cancer, inhibition of cell proliferation via vitamin D is associated with JNK1. JNK1 interacts with the VDR and regulates its expression, influencing  $1,25(\text{OH})_2\text{D}_3$ -mediated inhibition of proliferation of cancer cells [335]. Proliferating cells progress through the cell cycle, which comprises the G<sub>0</sub>/G<sub>1</sub> phase (most differentiated, nondividing cells are in the G<sub>1</sub> phase), the S phase in which new DNA is synthesized, and the G<sub>2</sub> phase, which is followed by mitosis (M phase) whereon the cells reenter the G<sub>0</sub>/G<sub>1</sub> phase. In most of the cells studied so far, treatment with  $1,25(\text{OH})_2\text{D}_3$  and its analogs results in a blockade at a specific checkpoint, i.e., the restriction point (R), in the G<sub>1</sub> phase limiting the transition of G<sub>1</sub> to S and reducing the number of cells in S phase. Some studies also have examined the effect on the G<sub>2</sub> phase, but these results are somewhat more diverse. In general, it can be concluded that blocking the transition from the G<sub>0</sub>/G<sub>1</sub> phase to the S phase plays an important role in the growth inhibitory effect of  $1,25(\text{OH})_2\text{D}_3$ . Numerous genes and proteins have been described that participate in the regulation of the cell cycle. It is beyond the scope of this chapter to discuss in detail the regulation of all of the genes/proteins by vitamin D. In Fig. 84.1, an overview is given of the interacting genes/proteins that are involved in intracellular signaling and regulating the cell cycle. These genes and proteins are part of the



cascade of events on which vitamin D exerts its effects. The components shown to be regulated by vitamin D are indicated. Fig. 84.1 is a compilation of data, and it is important to realize that probably not all of the genes/proteins are affected by vitamin D in all tumor cells. However, in this way, one can get an overview and appreciate the broad range of effects mediated by vitamin D on intracellular signaling pathways involved in regulation of (tumor) cell growth. More details on the regulation will be discussed in more detail in various other chapters in this section of the book especially Chapter 86. Overall, cell cycle checkpoint control is an interesting therapeutic target [336] where vitamin D alone or in combination with other compounds may act.

Related to cell cycle, checkpoints in DNA damage control [337,338]. Vitamin D has been implicated to be involved in control of genomic stability [339].  $1,25(\text{OH})_2\text{D}_3$  has been reported to inhibit hepatic chromosomal aberrations and DNA strand breaks [340]. This is supported by the finding that  $1,25(\text{OH})_2\text{D}_3$  and EB1089 stimulated the expression of GADD45, which stimulates DNA repair [341] and might be coupled to release of TP53 from Mdm2 (see Fig. 84.1). Notably, a recent study has shown that supplemental vitamin D<sub>3</sub> and calcium, separately but not together, decreased the level of the DNA damage marker 8-hydroxy-2'-deoxyguanosine in normal colorectal mucosa in a randomized clinical trial [342].  $1,25(\text{OH})_2\text{D}_3$  augmented DNA repair after UV-induced damage in skin cells [115,343,344], and other studies also provided information on vitamin D and DNA repair [255,345]. Tumor samples expressing low VDR harbored a unique set of genomic alterations compared with tumor samples expressing normal VDR levels [18].

### 3.3 Apoptosis, autophagy, and energy metabolism

The blockade in the cell cycle that prevents transition into S phase may cause cells to either go into apoptosis (programmed cell death) or enter a specific differentiation pathway. It is suggested that early G<sub>1</sub> phase may be the point at which switching between cell cycle progression and induction of apoptosis occurs [346,347]. Induction of apoptosis by  $1,25(\text{OH})_2\text{D}_3$  is an orderly and characteristic sequence of biochemical, molecular, and structural changes resulting in the death of the cell [348]. Apoptosis is a mechanism by which  $1,25(\text{OH})_2\text{D}_3$  inhibits tumor cell growth and may be the explanation for the tumor suppression and reduction in tumor volume found in various in vivo animal studies (see Section 2.2 and Table 84.2).

$1,25(\text{OH})_2\text{D}_3$  has been shown to regulate expression of apoptosis genes and to induce apoptosis of cancer

cells of various origins. For example,  $1,25(\text{OH})_2\text{D}_3$  and the analog Ro 25-6760 induce a cell cycle blockade in HT-29 human colon cancer cells causing growth inhibition and induction of apoptosis [349]. The *bcl-2* oncogene decreases the rate of programmed cell death [350]. However, protection of HL-60 cells against apoptosis occurred despite downregulation of *bcl-2* gene expression [351]. In several breast cancer cell lines (MCF-7, BT-474, MDA-MB-231),  $1,25(\text{OH})_2\text{D}_3$  and the analogs KH1060 and EB1089 decreased *bcl-2* expression [264,352] and also CB1093 reduced *bcl-2* expression in MCF-7 cells related to induction of apoptosis [353]. However, only in MCF-7 cells, this change in *bcl-2* expression was accompanied by apoptosis. The apoptosis induced by  $1,25(\text{OH})_2\text{D}_3$  and the analogs EB1089 and CB1093 in MCF-7 and T47D breast cancer cells does not involve caspases or TP53 activation [354].  $1,25(\text{OH})_2\text{D}_3$  induced apoptosis in MCF-7 cells via disruption of mitochondrial function, which is associated with Bax translocation to mitochondria, cytochrome *c* release, and production of reactive oxygen species [355]. It was shown that for MCF-7 cells, calpain, a calcium-dependent cysteine protease, may take over the role of the major execution protease in apoptosis-like death induced by vitamin D and EB1089 [356].

In B cell chronic lymphocytic leukemia cells in vitro (B-CLL), the vitamin D<sub>3</sub> analog EB1089 also induces apoptosis via a TP53-independent mechanism involving p38 MAP kinase activation and suppression of ERK activity [265]. In prostate cancer, the effects of vitamin D on apoptosis of tumor cells are caspase dependent and the human VDR is a target of caspase-3, suggesting that activation of caspase-3 may limit VDR activity [357].

Effects on other apoptosis genes/proteins such as BAX and BAK have been reported [358], and microarray gene expression analyses and differential screening will also definitively reveal additional vitamin D targets involved in regulating apoptosis [330,359]. Remarkably, treatment of patients with vitamin D<sub>3</sub> and calcium increased BAK immunostaining in the interior of colonic polyps [360] without affecting BCL2 expression in the same polyps [360] or in normal colon mucosa [361]. In a squamous cell carcinoma model system, the  $1,25(\text{OH})_2\text{D}_3$  analog inecalcitol showed antitumor activity via apoptosis through the activation of the caspase 8/10–caspase 3 pathway [362].

A central role for apoptosis in the action of  $1,25(\text{OH})_2\text{D}_3$  is unclear because growth inhibition of several other breast cancer cells besides MCF-7 cells appeared to be independent of apoptosis [264]. Also, MCF-7 cells that showed growth inhibition by  $1,25(\text{OH})_2\text{D}_3$  could, after removal of the hormone, again be stimulated to grow, implying transient growth inhibition and not cell death [244]. Stable transfection of leukemic U937 cells with the wild-type TP53 tumor



suppressor gene resulted in a reduced growth rate and produced cells that can undergo either apoptosis or maturation. In these cells,  $1,25(\text{OH})_2\text{D}_3$  protects against TP53-induced apoptosis and enhances TP53-induced maturation [262]. In two independent studies with HL-60 cells,  $1,25(\text{OH})_2\text{D}_3$  was found either to protect against or to have no effects on apoptosis [351,363]. Vitamin D protection against apoptosis was also detected in human U937 leukemic cells treated with tumor necrosis factor  $\alpha$  [364]. Absence of a vitamin D effect on apoptosis might be explained by the expression of the antiapoptotic protein BAG-1 p50 isoform. This protein has been shown to bind to the VDR and block vitamin D–induced transcription [365]. Presence of additional interacting factors might also be important for the eventual effect on apoptosis as in the study with HL-60 cells where, in the presence but not the absence of 9-cis-retinoic acid,  $1,25(\text{OH})_2\text{D}_3$  did induce apoptosis [363]. Role of vitamin D interaction with other factors will be discussed in more detail in Section 4. Over the years, an increasing number of studies on vitamin D and apoptosis are reported. While most studies show an increase in apoptosis also, studies report inhibition of apoptosis. This latter may be related to the effect of vitamin D on another process: autophagy.

Autophagy is a process by which cells direct their components to the lysosome via autophagosomes for degradation, and it plays an important role in tissue homeostasis. The term autophagy originates from the 1950s when it was introduced by Christian de Duve. The Nobel Prize for Physiology or Medicine in 2016 for discoveries of the mechanisms of autophagy can be considered as an indication of the significance of this process. Autophagy has been linked to various diseases, including cancer [366,367]. The role of autophagy in cancer is not unequivocal. Both stimulation and inhibition of autophagy have been proposed in cancer therapy. Several reports show that vitamin D affects autophagy, either stimulating [368,369], or VDR-mediated prevention of prosurvival autophagy [370]. The eventual effect is thought to be tumor/cancer cell context dependent.

Apoptosis and autophagy are different but related processes. The relationship is nicely summarized by Eisenber-Lerner et al. [371]. The literal citation of their conclusion is: “The cross-talk between apoptosis and autophagy is therefore quite complex, and sometimes contradictory, but surely critical to the overall fate of the cell. Furthermore, the cross-talk is a key factor in the outcome of death-related pathologies such as cancer, its development and treatment.” The relationship and/or balance between apoptosis and autophagy can also be seen for vitamin D. Several studies report vitamin D or vitamin D analog inhibition of apoptosis together with stimulation of autophagy [372–375].

A fundamental process of cellular function is energy metabolism. In normal cells, ATP is formed by a multi-step degradation (TCA cycle) of glucose and oxidative phosphorylation, which takes place in the mitochondria and is dependent on oxygen. Cancer cells can display metabolic reprogramming and employ a phenomenon called Warburg effect/aerobic glycolysis, i.e., glucose fermentation and production of lactate independent of ambient oxygen levels. This generates less energy than mitochondrial oxidative phosphorylation, but tumor cells often compensate by increasing glucose uptake by glucose transporters [376,377]. This increased glucose uptake is at the basis of cancer imaging, FDG-PET scanning. Considering the importance of energy metabolism for cancer cell growth, it is a target for therapeutic intervention. A role for vitamin D in the control of energy metabolism is conceivable from an evolutionary perspective [378]. Vitamin D and cancer cell energy metabolism has been extensively discussed by Abu el Maaty et al. and Sheeley et al. [379,380]. Vitamin D in combination with testosterone is reported to affect the TCA cycle by regulating gene expression [381]. Vitamin D–induced decrease in expression of glucose transporter and reduction in glycolysis has been linked to decreased migration by breast cancer cells [382]. Inhibition of the Warburg effect by vitamin D has been reported in non–small-cell lung cancer [383] and colorectal cancer cells [384]. A study in colorectal cancer cells brings together the regulation of an oncogene, regulatory noncoding RNA, energy metabolism, and vitamin D. A long noncoding RNA induced by vitamin D suppresses glycolysis via enhanced degradation of cMyc [385]. However, vitamin D augmented repair of UV-induced DNA damage in skin cells is linked to an increased energy availability [344]. This indicates that the eventual cellular response, e.g., proliferation, differentiation, repair, is important to take in account.

In summary, the data obtained so far show that  $1,25(\text{OH})_2\text{D}_3$ -induced growth inhibition can be related to apoptosis in some cases, but that growth inhibition also can be observed independent of apoptosis. Possibly in these latter cases, induction of autophagy and/or differentiation is more prominent. The factor(s) that decide whether cells undergo apoptosis or go another route like differentiation is(are) unclear but is probably dependent on cell cycle stage, DNA damage, presence of other factors, and levels of expression of various oncogenes and tumor suppressor genes. These variables contribute to what appears to be cell-specific actions of vitamin D to induce apoptosis and/or autophagy. Here also the cancer cell context in the tumor (stromal and immune cells) is important for the eventual effect. An interesting phenomenon to be studied concerning vitamin D and apoptosis is calbindin 28K. Calbindin 28K is a well-known vitamin D–induced protein, which has recently

been shown to inhibit apoptosis [386]. It is tempting to speculate that calbindin 28K plays a role in the decision whether vitamin D induces cells to differentiate or to go into apoptosis or that it is involved when  $1,25(\text{OH})_2\text{D}_3$  protects against apoptosis. Additionally, EB1089 induces lysosomal changes and autophagic cell death in human MCF-7 breast cancer cells [387,388]. Finally, energy metabolism is an important target for vitamin D in cancer cells where various cellular effects of vitamin D may converge and which deserves extensive research in the future.

### 3.4 Cell differentiation

Differentiation is another cellular process in the array of vitamin D anticancer actions. There is a considerable body of evidence that human cancer cells can be suitable candidates for differentiation therapy with vitamin D [183]. However, mechanisms of  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation are cell-type and cell-context specific [389,390]. The coupling between proliferation and differentiation has been most widely studied for cells of the hematopoietic system and keratinocytes. In general,  $1,25(\text{OH})_2\text{D}_3$  inhibits proliferation and induces differentiation along the monocyte–macrophage lineage. Rapidly proliferating and poorly differentiated keratinocytes can be induced to differentiate by  $1,25(\text{OH})_2\text{D}_3$ . A further relationship between the vitamin  $\text{D}_3$  system and differentiation is demonstrated by the fact that in poorly differentiated keratinocytes,  $1,25(\text{OH})_2\text{D}_3$  production and vitamin D receptor levels are high, whereas after induction of differentiation, these levels decrease [391]. In melanoma cells, in addition to growth inhibition [84],  $1,25(\text{OH})_2\text{D}_3$  stimulates melanin production [392]. Effects on differentiation have also been reported for other cell types. Inhibition of prostate cancer cell proliferation is paralleled by an increased production of PSA per cell, a sign of differentiation [393,394]. In the BT-20 breast cancer cells,  $1,25(\text{OH})_2\text{D}_3$  induced morphological changes indicative for differentiation [395]. In triple-negative breast cancer,  $1,25(\text{OH})_2\text{D}_3$  inhibits cancer stem-like cells and induces differentiation [396]. In several breast cancer cell lines, the stimulation of differentiation has been established by determining lipid production by the cells [264]. In this study, Elstner et al. demonstrated an uncoupling between effects on proliferation and differentiation. In two breast cancer cell lines,  $1,25(\text{OH})_2\text{D}_3$  and various analogs induced differentiation even though the cells were resistant to cell cycle and antiproliferative effects. This together with data obtained with human myelogenous leukemia cells [257] suggests a dissociation between the cellular vitamin  $\text{D}_3$  pathways involved in regulation of differentiation and proliferation (see also Section 5).

For an HL-60 subclone, a similar observation was made [239], and in another HL-60 subclone, the induction of differentiation was found to precede the  $\text{G}_0/\text{G}_1$  cell cycle blockade. In contrast to the aforementioned observations on stimulation of differentiation,  $1,25(\text{OH})_2\text{D}_3$  inhibits erythroid differentiation of the erythroleukemia cell line K562 [397], and  $1,25(\text{OH})_2\text{D}_3$  inhibits activin A-induced differentiation of murine erythroleukemic F5-5 cells [398]. Paracalcitol, a vitamin  $\text{D}_2$  analog, converted committed myeloid hematopoietic stem cells from wild-type but not from VDR knockout mice to differentiate into macrophages [399]. Osteoclast-like differentiation of malignant plasma cells is enhanced by  $1,25(\text{OH})_2\text{D}_3$  [400].

Shabahang et al. [2] found that the VDR level correlated with the degree of differentiation in human colon cancer cell lines and suggested it might serve as a useful biological marker in predicting clinical outcome in patients. Differentiation of rapidly dividing HT-29 colon cancer cells to differentiated slowly proliferating cells was associated with decreased VDR abundance, loss of VDR homologous upregulation, and the development of hormone unresponsiveness to  $1,25(\text{OH})_2\text{D}_3$  [351].  $1,25(\text{OH})_2\text{D}_3$  induces an adhesive phenotype typical of the differentiated epithelial cells that is mostly based on the upregulation of E-cadherin and other plasma membrane adhesion proteins of adherens junctions ( $\alpha$ -catenin) and tight junctions (occludin, claudins, ZO-1) [243,401]. Also,  $1,25(\text{OH})_2\text{D}_3$  regulates the phenotype of human breast cancer cells. Thus, it increases the expression of E-cadherin, claudin-7, and occludin and of proteins such as paxillin, focal adhesion kinase, and  $\alpha\text{v}$ - and  $\beta 5$ -integrins that are involved in adhesion to the substratum [402]. Moreover,  $1,25(\text{OH})_2\text{D}_3$  represses several markers of the basal/myoepithelial phenotype (P-cadherin, smooth muscle  $\alpha$ -actin, and  $\alpha 6$  and  $\beta 4$ -integrins), the proinvasive and proangiogenic protein tenascin-C protein, and the mesenchymal marker N-cadherin that are associated with aggressiveness and poor prognosis in breast cancer [403,404]. The effect of vitamin D on the epithelial–mesenchymal transition can also be considered related to differentiation or in other words, retaining the differentiation status.

### 3.5 Growth factors and growth factor receptors

The interaction with tumor- or stroma-derived growth factors is important for the action of vitamin D. Stimulation of breast cancer cell proliferation by coculture with fibroblasts is inhibited by  $1,25(\text{OH})_2\text{D}_3$  [405]. A good candidate to interact with the  $1,25(\text{OH})_2\text{D}_3$  action is transforming growth factor- $\beta$  (TGF $\beta$ ). TGF $\beta$  is involved in cell cycle control and apoptosis [406,407]. TGF $\beta$  can interfere with the cascade of events in the GI

phase described before and inhibit the ability of cells to enter S phase when it is present during the G1 phase. TGF $\beta$  has been shown to suppress *c-myc*, cyclin A, cyclin E, and cdk2 and cdk4 expression [407]. In line with this, TGF $\beta$  has been reported to inhibit phosphorylation of p110<sup>RB</sup> [408]. Vitamin D<sub>3</sub> compounds induce dephosphorylation of the retinoblastoma gene product, and vitamin D<sub>3</sub> growth inhibition of MCF-7 breast cancer cells is inhibited by a TGF $\beta$  neutralizing antibody [409]. 1,25(OH)<sub>2</sub>D<sub>3</sub> and several analogs stimulated the expression of TGF $\beta$  mRNA and secretion of active and latent TGF $\beta$ 1 by the breast cancer cell line BT-20 [176]. 1,25(OH)<sub>2</sub>D<sub>3</sub> enhanced TGF $\beta$ 1 gene expression in human keratinocytes [410] and the secretion of TGF $\beta$  in murine keratinocytes [411]. In both studies, antibodies against TGF $\beta$  inhibited the growth inhibitory effect of vitamin D<sub>3</sub>.

Further evidence for a vitamin D<sub>3</sub>–TGF $\beta$  interaction is that bone matrix of vitamin D–deficient rats contains substantially less TGF $\beta$  than controls [412]. It has been shown for the interaction between TGF $\beta$  signaling pathways and vitamin D that the cross-talk may be mediated by Smad3. Smad3, one of the SMAD proteins downstream in the TGF $\beta$  signaling pathway, was found in mammalian cells to act as a coactivator specific for ligand-induced transactivation of VDR by forming a complex with a member of the steroid receptor coactivator-1 protein family in the nucleus [413]. However, Smad3 is not of itself sufficient to coactivate VDR in TGF $\beta$ /vitamin D<sub>3</sub>-resistant MCF7L cells, and other factors are required. It was found that the PI 3-kinase pathway inhibitor LY29004 inhibited the synergy of TGF $\beta$  and EB1089 on VDR-dependent transactivation activity. This indicates that the cross-talk between TGF $\beta$  and vitamin D signaling is also PI 3-kinase pathway dependent [414]. Therefore, on the basis of these consistent findings, TGF $\beta$  is a likely candidate to play a role in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced growth inhibition [414]. Gene expression profiling of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated normal and breast cancer cells identified enrichment of TGF $\beta$  signaling pathway [332].

Interactions with the insulin-like growth factor [266] system have also been described. IGFs are potent growth stimulators of various cells, and their effect is regulated via a series of IGF binding proteins (IGFBPs). The IGFBPs, especially IGFBP-3, have potent antiproliferative and proapoptotic actions [415]. These effects include both IGF-dependent actions, by sequestering the potent growth factor, and IGF-independent, having direct actions via its own receptor [416,417]. Among the many ways, vitamin D inhibits prostate cancer growth; stimulation of IGFBP-3 may be a major contributor [418]. 1,25(OH)<sub>2</sub>D<sub>3</sub> and the analog EB1089 inhibit the IGF-I-stimulated growth of MCF-7 breast cancer cells [419]. In prostate cancer cell lines, 1,25(OH)<sub>2</sub>D<sub>3</sub> induced

expression of IGFBP-6 but not IGFBP-4 [420]. In human osteosarcoma cell lines, 1,25(OH)<sub>2</sub>D<sub>3</sub> and the analog 1 $\alpha$ -dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol potently stimulated the expression and secretion of IGFBP-3 [421–423]. In one study, an association has been made between increased IGFBP-3 levels and 1,25(OH)<sub>2</sub>D<sub>3</sub> growth inhibition [421]. Recent observations that antisense oligonucleotides to IGFBP-3 prevented growth inhibition of prostate cancer cells by 1,25(OH)<sub>2</sub>D<sub>3</sub> [329] provided further evidence for an interplay between 1,25(OH)<sub>2</sub>D<sub>3</sub> and IGFBP-3. Interestingly, in the human osteosarcoma cell line, MG-63, 1,25(OH)<sub>2</sub>D<sub>3</sub> and TGF $\beta$  synergistically increased IGFBP-3 secretion [423]. A study published in 2021 underscores the interaction between 1,25(OH)<sub>2</sub>D<sub>3</sub> and TGF $\beta$  by demonstrating a synergistic stimulation of IGFBP3 expression [424]. In a randomized double-blind placebo-controlled 12-month study of 20,000 IU cholecalciferol orally per week, no differences in serum IGF-I and in IGFBP3 between vitamin D and placebo were detected [425]. Unfortunately, no data on intratumor concentrations were available, as these might be more insightful than circulating levels.

IGF-II is also a growth and survival factor for colorectal cancer cells, and 1,25(OH)<sub>2</sub>D<sub>3</sub> and several analogs interfere with IGF-II signaling. They upregulate IGFBP-6, which inhibits IGF-II signaling, and type II IGF receptor (IGF-R-II) that also blocks this pathway and accelerates IGF-II degradation [426,427]. An example of growth factor receptor regulation by 1,25(OH)<sub>2</sub>D<sub>3</sub> concerns the epidermal growth factor receptor (EGFR). This receptor is downregulated in T47-D breast cancer cells and upregulated in BT-20 breast cancer cells. Nevertheless, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the growth of both cell lines [428,429]. The 1,25(OH)<sub>2</sub>D<sub>3</sub> growth inhibition was strongest in lung cancer cells with a mutated EGFR [430]. These data provide evidence that interactions with growth factors are part of the 1,25(OH)<sub>2</sub>D<sub>3</sub> action on tumor cells. In primary colon adenocarcinoma cells as well as in the colon cancer Caco-2 cell line, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits EGF mitogenic signaling, and a mutual modulation of receptor expression between 1,25(OH)<sub>2</sub>D<sub>3</sub> and EGF has been proposed [431,432]. In A431 epidermoid cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> alters EGFR membrane trafficking and inhibits EGFR signaling [433].

It was found that TCF-4, a transcriptional regulator and beta-catenin binding partner, is an indirect target of the VDR pathway. TCF-4 functions as a transcriptional repressor that restricts breast and colorectal cancer cell growth. 1,25(OH)<sub>2</sub>D<sub>3</sub> increases TCF-4 at the RNA and protein levels in several human colorectal cancer cell lines, which is completely dependent on the VDR. This 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated increase in TCF-4 may have a protective role in colon cancer as well as other diseases [434]. In ovarian cancer,

1,25(OH)<sub>2</sub>D<sub>3</sub> decreases the expression of lncRNA CCAT2, and by blocking the CCAT2–TCF-4 interaction, it reduces c-Myc expression and inhibits the binding to TCF-4, which is paralleled by a decrease in growth and migration [435]. In an *in vivo* model of liver tumor formation, vitamin D deprivation caused tumor growth in the context of TGFβ/Smad3 disruption, via regulation of Toll-like receptor 7 expression and β-catenin activation [436].

As described before, it is clear that 1,25(OH)<sub>2</sub>D<sub>3</sub> has effects on the transcription of numerous RNA species, including coding and noncoding RNAs, and multiple interactions with various growth factors exist. With respect to growth inhibition, at this time, two models of action can be postulated. In the first one, 1,25(OH)<sub>2</sub>D<sub>3</sub> directly interferes with a crucial gene(s) involved in the control of the cell cycle. In this case, in view of the general pattern of the genes involved in cell cycle control, this mechanism of action will be similar in all types of cancer cells. However, the effect on cell cycle genes will be dependent on contextual parameters such as tumor stroma, immunological makeup, and thereby the presence or absence of additional growth factors and cytokines. This will eventually determine the differences in 1,25(OH)<sub>2</sub>D<sub>3</sub> action between cancer types of different origin but also within cancer types of similar origin. In the second model, 1,25(OH)<sub>2</sub>D<sub>3</sub> may regulate cell cycle indirectly via changing the production of regulatory and signaling molecules such as growth factors, growth factor signaling, growth factor–binding protein levels, or receptor regulation. It is conceivable that a combination of both models forms the basis of 1,25(OH)<sub>2</sub>D<sub>3</sub> regulation of tumor cell growth. It is conceivable that the consequences, e.g., apoptosis, autophagy, differentiation, of cell cycle interference are dependent on the mutational profile of the cancer cell and the tumor microenvironment. A complicating aspect herein is the tumor heterogeneity [15,16].

#### 4. Combination therapy

The data obtained with 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs on growth inhibition and stimulation of differentiation offer promise for their use as an anticancer treatment. Single agent treatment with low calcemic 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs could be useful; however, combination therapy with other tumor effective drugs may provide an even more beneficial effect [156]. More and more studies are focusing on combination therapies with 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs.

Initially, most combination studies focused on breast cancer cells and the combination of one of the most widely used endocrine therapies, the antiestrogen tamoxifen, with 1,25(OH)<sub>2</sub>D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs.

This combination resulted in a greater growth inhibition of MCF-7 and ZR-75-1 cells than treatment with either compound alone [132,437]. In combination with tamoxifen, the cells were more sensitive to the antiproliferative action of 1,25(OH)<sub>2</sub>D<sub>3</sub> and the analogs; that is, the EC<sub>50</sub> values of the vitamin D<sub>3</sub> compounds in the presence of tamoxifen were lower than those in the absence of tamoxifen [244]. Vitamin D induces tamoxifen sensitivity in MCF-7 cells via inhibition of Wnt signaling. Studies with MCF-7 cells suggested a synergistic effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> and tamoxifen on apoptosis [438]. In addition, in *in vivo* breast cancer models, a synergistic effect of the tamoxifen-1,25(OH)<sub>2</sub>D<sub>3</sub> analogs combination was observed [132,133].

Another interesting interaction relevant to breast cancer is that vitamin D inhibits aromatase, thus reducing the estrogenic stimulus for proliferation [311]. Combination of 1,25(OH)<sub>2</sub>D<sub>3</sub> and aromatase inhibitors also showed synergistic activity in breast cancer cells. 1,25(OH)<sub>2</sub>D<sub>3</sub> also downregulates the estrogen receptor, again reducing the ability of estrogens to stimulate breast cancer growth [439]. Additional data on the interaction between the estrogen/antiestrogen system and vitamin D come from studies showing the presence of an estrogen-responsive element in the VDR promoter and regulation of VDR by estradiol in breast cancer cells [440]. It is intriguing that the stimulator of breast cancer cell growth induces the expression of the receptor for a growth inhibitor. VDR upregulation in breast cancer cells and increased transcriptional activity was mimicked by the phytoestrogens resveratrol and genistein and blocked by tamoxifen [441]. Interaction between the estrogen system and CYP24A1 is also of importance. Data have shown that genistein inhibits CYP24A1 activity in prostate cancer cells and thereby increases the responsiveness to 1,25(OH)<sub>2</sub>D<sub>3</sub> [442,443]. The combination of genistein and cholecalciferol has been tested in a phase II randomized placebo-control trial for prostate cancer, but the results were inconclusive [444].

Estradiol induces metastasis-associated protein (MTA)-3, a component of the Mi-2/NuRD transcriptional corepressor complex that inhibits Snail1, which is in turn a repressor of VDR gene expression [445,446]. In this way, estradiol may increase VDR levels in breast cells. In colon cancer also, VDR upregulation by estradiol has been reported; however, in colon, it was hypothesized to contribute to the protective effect of estradiol on chemically induced colon carcinogenesis [447]. These interactions between the vitamin D and estrogen endocrine systems in the regulation of cancer [310] are promising and warrant further detailed analyses, e.g., regarding tissue (cancer)-specific effects. Interaction with another sex steroid, testosterone, has been described for ovarian cancer. Vitamin D inhibits



dihydrotestosterone (not convertible to estradiol) growth stimulation of ovarian cancer cells [448]. Intriguingly, also here the growth stimulator and growth inhibitor mutually upregulate their receptors. Triple-negative breast cancer can be targeted with androgen receptor (AR) and/or VDR agonists to reduce viability of cancer cells and to change in cancer stem cell phenotype. The combination of AR and VDR agonists with chemotherapy was additive [449]. In prostate cancer cells, it has been shown that  $1,25(\text{OH})_2\text{D}_3$  while inhibiting androgen stimulated growth upregulates the androgen receptor [450].

Interaction with another steroid in regulating cancer cells has already been reported in 1983. The synthetic glucocorticoid dexamethasone and  $1,25(\text{OH})_2\text{D}_3$  synergistically induced differentiation of murine myeloid leukemia cells [451]. This was supported by in vitro and in vivo data that dexamethasone enhanced the effect of vitamin D on growth inhibition, cell cycle arrest, and apoptosis of squamous carcinoma cells [452,453]. A possible mechanism is the upregulation of VDR by dexamethasone [452]. An interesting aspect of this combination is not only the direct interaction at cancer cell level but also in the control of the calcemic action of  $1,25(\text{OH})_2\text{D}_3$ . Glucocorticoids inhibit intestinal calcium absorption and increase renal calcium excretion, and in this way, it may limit the hypercalcemic action of  $1,25(\text{OH})_2\text{D}_3$  [454].

Combinations of vitamin D and retinoids have been examined in various systems. A combination of retinoic acid and  $1,25(\text{OH})_2\text{D}_3$  resulted in a more profound growth inhibition of both T47-D breast cancer cells [455] and LA-N-5 human neuroblastoma cells [456]. 9-cis-Retinoic acid augmented  $1,25(\text{OH})_2\text{D}_3$ -induced growth inhibition and differentiation of HL-60 cells [457]. Besides growth inhibition and differentiation effects, the combination of  $1,25(\text{OH})_2\text{D}_3$  and various isomers of retinoic acid was more potent in reducing angiogenesis than either compound alone [167,458,459]. The background of the interaction between retinoids and  $1,25(\text{OH})_2\text{D}_3$  may be attributed to heterodimer formation of their respective receptors [460].

For several cytokines, interactions with  $1,25(\text{OH})_2\text{D}_3$  have been described, stressing the importance of immune system and vitamin D relationship in cancer [461,462]. Interferon- $\gamma$  and  $1,25(\text{OH})_2\text{D}_3$  synergistically inhibited the proliferation and stimulated the differentiation of myeloid leukemia cells [463]. Treatment of LLC-LN7 tumor cells with  $1,25(\text{OH})_2\text{D}_3$  and IFN- $\gamma$  synergistically reduced tumor granulocyte-macrophage colony-stimulating factor (GM-CSF) secretion and a blockage in the capacity of the tumor cells to induce granulocyte-macrophage-suppressor cells [103]. In the mouse myeloid leukemia cells,

interleukin-4 enhanced  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation [464]. Also with interleukin-1 $\beta$ , interleukin-3, interleukin-6, and interleukin-12, interactions with  $1,25(\text{OH})_2\text{D}_3$  have been reported [465–467]. Although a small sample size (43 cases vs. 86 controls), in clear cell renal cell carcinoma patients but not in controls serum, interleukin-6 and  $25(\text{OH})\text{D}$  levels were inversely correlated. pSTAT3 signal was significantly higher in cancerous tissue from  $25(\text{OH})\text{D}$ -deficient (<20 mg/mL) than in  $25(\text{OH})\text{D}$ -insufficient and  $25(\text{OH})\text{D}$ -sufficient groups (>20 mg/mL) [468].  $1,25(\text{OH})_2\text{D}_3$  and tumor necrosis factor synergistically induced growth inhibition and differentiation of HL-60 [469]. For MCF-7 cells, an interaction between  $1,25(\text{OH})_2\text{D}_3$  and tumor necrosis factor has also been reported [467,470]. In the presence of GM-CSF, lower concentrations of  $1,25(\text{OH})_2\text{D}_3$  could be used to achieve a similar antiproliferative effect in MCF-7 cells [471] and to induce differentiation of U937 myeloid leukemic cells [472].

Recently, interactions with a plant-derived polyphenolic antioxidant, carnosic acid, were demonstrated in the differentiation of HL-60 cells, which was related to a decrease in the intracellular levels of reactive oxygen species [473,474].  $1,25(\text{OH})_2\text{D}_2$  in combination with a plant polyphenol is a potent inducer of differentiation in acute myeloid leukemia cells [475]. Other factors shown to interact with  $1,25(\text{OH})_2\text{D}_3$  are butyrate [476,477], melatonin [478], and other factors, such as EGFR, described in Section 3.5. The EGFR signals via the tyrosine kinase pathway. Vitamin D enhances the effect of tyrosine kinase inhibitors [479] also in a triple combination with cytostatic drugs [480]. The relationship between vitamin D and LXR [21] requires further studies as the combination of vitamin D and LXR ligand T0901317 is more potent in inducing apoptosis in breast cancer cells [481].

The so far described studies are combination of vitamin D with other hormones and/or bioactive compounds (growth factors and cytokines) where the mechanism of combination may lie in convergence and interaction of their respective signaling pathways and can be primarily cancer cell specific. Interestingly, also for two other, broadly used cancer therapies interactions with vitamin D have been observed: first, the combination of chemotherapy/cytostatic drugs and vitamin D. In vivo adriamycin and in vitro carboplatin, cisplatin, and doxorubicin interacted synergistically with  $1,25(\text{OH})_2\text{D}_3$  to inhibit breast cancer cell growth [127,482–484]. In a carcinogen-induced rat mammary tumor model, treatment with  $1\alpha-(\text{OH})\text{D}_3$  and 5-fluorouracil, however, did not result in enhanced antitumor effects [100]. In other cancer types, enhancement of chemotherapy has been described [485–488]. Unwanted side effects are often seen with the use of cytostatics. Vitamin D together

with pirfenidone limits chemotherapy induced renal toxicity in mice [489].

Secondly, the combination of vitamin D and radiotherapy is under investigation. Vitamin D enhances the effect of radiation therapy in various cancer cell types [490–494]. A combination of vitamin D with photodynamic therapy to enhance tumor cell death has also been reported [495,496].

Combination studies showed that vitamin D is sensitizer for proton therapy [497] and cryoablation [498–500]. The mechanism(s) by which vitamin D enhances or sensitizes these chemotherapy and radiotherapy are generally unknown, but it may involve effects on the immune system. Two studies on combination with radiotherapy point in this direction [501,502].

It is known that food components can impact the efficacy of cancer therapies. For example, the herb curcumin negatively affects the pharmacokinetics of tamoxifen [503] while the acid beverage cola positively affects the pharmacokinetics of erlotinib by increasing the bioavailability [504]. For vitamin D also, interaction with food components curcumin [505,506] and S-adenosylmethionine has been reported. Intake of food or beverage components may impact vitamin D metabolism. Chronic intake of ethanol regulates CYP27B1 and CYP24A1 in a breast cancer mouse model [507].

A development over the recent years is a novel way of delivery of vitamin D in combination with other compounds. Vitamin D is applied together with cisplatin, paclitaxel, doxorubicin, or etoposide in nanoparticles or micelles [508–511]. Also micelles have been generated that both in vitro and in vivo targetedly delivered high dosages of vitamin D at breast cancer cells and caused growth and metastasis inhibition [512].

The data on combinations of  $1,25(\text{OH})_2\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  analogs with various other anticancer compounds and therapies are promising and merit further analyses. The development of effective combination therapies may result in better response rates and lower required dosages, thereby reducing the risk of negative side effects. An additional benefit is that some direct actions of  $1,25(\text{OH})_2\text{D}_3$  may reduce side effects of toxic chemotherapy drugs when given in combination [513]. An overview and possibilities of combined cancer treatments of vitamin D and other compounds is given by Gocek and Studzinski [514]. The development of novel tools of drug delivery opens novel avenues for combination and targeted therapies.

## 5. Resistance and vitamin D metabolism

We will discuss first vitamin D resistance of tumor cells and second more general development of therapy resistance of tumors and cancer cells. Classic vitamin

D resistance concerns the disease hereditary vitamin D-resistant rickets, which is characterized by the presence of a nonfunctional VDR and consequently aberrations in calcium and bone metabolism (see Chapter 68). For cancer cells, the presence of a functional VDR is a prerequisite for a growth regulatory response, and a relationship between VDR level and growth inhibition has been suggested for osteosarcoma, colon carcinoma, breast cancer, prostate cancer cells, and rat glioma [263,515–518]. Cell lines established from DMBA-induced breast tumors in VDR knockout mice are insensitive to growth arrest and apoptosis by  $1,25(\text{OH})_2\text{D}_3$ , EB1089, and CB1093 [519]. Albeit that VDR is a prerequisite for tumor cell growth regulation, the presence of the VDR is not always coupled to a growth inhibitory response of  $1,25(\text{OH})_2\text{D}_3$ . Results from studies with transformed fibroblasts [246], myelogenous leukemia cells [239,257,520], transformed keratinocytes [521], and various breast cancer cell lines [264,522] demonstrated a lack of growth inhibition by  $1,25(\text{OH})_2\text{D}_3$  even in the presence of VDR. In this situation, the designation “resistant” is based on the lack of growth inhibition, even though, as discussed earlier in Section 3.4, some of these cells are still capable of being induced to differentiate [257,264]. This points to a specific defect in the growth inhibitory pathway. In the resistant MCF-7 cells, this defect is not located at a very common site in the growth inhibitory pathway of the cell, because the growth could still be inhibited with the antiestrogen tamoxifen [522]. For myelogenous leukemia cells, similar observations have been made [523]. Human VDR gene is transcriptionally repressed by SNAIL1 and SNAIL2/SLUG in human colon cancer cells, leading to decreased levels of VDR RNA and protein and unresponsiveness to  $1,25(\text{OH})_2\text{D}_3$  effects [524–526]. SNAIL1 causes also a decrease in VDR RNA stability [524]. Also, Snail1 represses VDR in mouse osteoblasts and SNAIL2/SLUG in human breast cancer cells [527]. In addition, Snail1 is probably mediating the decrease in VDR mRNA stability induced by oncogenic Ha-ras in mouse NIH-3T3 cells [528,529]. Treatment of HT-29 colonic carcinoma cells with the flavonolignan silibinin reduced SNAIL expression and restored VDR transcription and  $1,25(\text{OH})_2\text{D}_3$  growth inhibition [530]. Hypermethylation of about 700 base pairs upstream as well as close to the transcription start site of the VDR gene has been demonstrated and linked to reduced VDR expression and VDRE containing genes such C/EBP and p21 [531]. In cancerous tissue, the frequency of VDR promoter methylation was about four times higher than in normal tissue, and methylation status was significantly associated with tumor staging [532].

For VDR-independent resistance to growth inhibition and in general to  $1,25(\text{OH})_2\text{D}_3$  effects, several underlying mechanism(s) have been proposed, such as (1)

increased levels of VDR corepressors, (2) reduced bioavailability of  $1,25(\text{OH})_2\text{D}_3$  due to CYP24A1 upregulation and/or CYP27B1 downregulation, and increased 24-hydroxylase or decreased 25-hydroxyvitamin D3  $1\alpha$ -hydroxylase activity, respectively, and (3) disruption or phosphorylation of VDR-RXR dimers. Resistance to  $1,25(\text{OH})_2\text{D}_3$  in breast and prostate cancer cells has also been found to be a consequence of increased levels of the VDR corepressors NCoR or SMRT [533,534]. This is in line with the reported synergistic effect on the proliferation of prostate cancer cells of combined treatment with  $1,25(\text{OH})_2\text{D}_3$  and the histone deacetylase inhibitor trichostatin [476].

For the resistant MCF-7 clone, this is not related to upregulation of the P-glycoprotein [522]. Interestingly, these vitamin D-resistant MCF-7 clones can be sensitized to vitamin D by activation of protein kinase C, resulting in induction of apoptosis and transcriptional activation, suggesting that alterations in phosphorylation may affect vitamin D sensitivity [535]. Hansen et al. [536] described an interesting growth inhibition-resistant MCF-7 cell clone. This clone was not growth-inhibited, while VDR was still present and CYP24A1 could still be induced.

In the resistant leukemia JMMD3 cell line, altered regulation and DNA binding activity of *junD* as part of the AP-1 complex has been reported [252]. Resistance to growth inhibition in the presence of VDR has also been linked to disruption of the VDR-RXR complex [537] and increased RXR degradation [538]. In addition, other factors, such as the acute myeloid leukemia translocation products (e.g., PLZF), may contribute to resistance to vitamin D by sequestering the VDR [288,289]. More recently, it was shown that altered corepressor and coactivator interaction with VDR and that epigenetic preferential suppression of antiproliferative gene promoters can explain the resistance to growth inhibition [539]. Resistance has also been linked to epigenetic changes in the VDR promoter, leading to suppressed or absent expression of VDR.

A unique mechanism for vitamin D resistance in immortalized cells has very recently been uncovered. Epstein-Barr virus (EBV) has been used to transform and immortalize lymphoblasts that can grow as cell lines in vitro. EBNA3 is an EBV encoded protein that can regulate transcription of cellular and viral genes. EBNA3 binds the VDR and blocks the activation of VDR-dependent genes and protects transformed cell lines against  $1,25(\text{OH})_2\text{D}_3$ -induced growth arrest and/or apoptosis [540]. The  $1,25(\text{OH})_2\text{D}_3$ -sensitive and resistant cell clones provide interesting models to examine the molecular mechanisms of  $1,25(\text{OH})_2\text{D}_3$ -induced growth inhibition. For example, lack of p21 results in no cell cycle block [541], and no apoptosis was detected with a mutated TP53 [264]. The identification of cellular

proteins that are involved in the vitamin D resistance in new world primates might add to the understanding of tumor cell resistance to vitamin D [542,543].

An important mechanism for vitamin D resistance or reduced sensitivity in VDR containing tumor and cancer cells is  $1,25(\text{OH})_2\text{D}_3$  catabolism via the C24-hydroxylation pathway. An inverse relationship between cellular metabolism of  $1,25(\text{OH})_2\text{D}_3$  via 24-hydroxylation and growth inhibition of prostate cancer cells has been suggested [516]. The latter observation is intriguing, the more so as an inverse relationship between VDR level and induction of CYP24A1 activity was reported. In general, there is a direct relationship between VDR level and induction of CYP24A1 activity [517,544].

A role for CYP24A1 in the control of  $1,25(\text{OH})_2\text{D}_3$  action on cancer cells was provided by studies with the  $1,25(\text{OH})_2\text{D}_3$ -resistant prostate cancer cell line DU145. It was shown that  $1,25(\text{OH})_2\text{D}_3$  did inhibit the growth of these cells when it was combined with the 24-hydroxylase inhibitor Liazorole [545].  $1,25(\text{OH})_2\text{D}_3$  activity was likewise enhanced by combination with ketoconazole, a drug commonly used to treat prostate cancer that inhibits CYP24A1 activity [546,547]. Inhibition of CYP24A1 activity in HL-60 cells also altered the effect of  $1,25(\text{OH})_2\text{D}_3$  and 20-epi analogs [548]. CYP24A1-mediated reduction in  $1,25(\text{OH})_2\text{D}_3$  stimulated self-renewal of stem cell-like glioma cells [549]. Recently, epigenetic silencing of the CYP24A1 gene modulates the growth response of tumor-derived endothelial cells [550]. Selective removal of CYP24A1 in mouse mammary epithelium increases vitamin D sensitivity and reduces proliferation [551], and breast cancer growth is stimulated by CYP24A1-induced vitamin D deficiency [552]. The action of the analog EB1089 was also limited by hydroxylation at the C24 position [553]. However, it was suggested that the increased potency of EB1089 is at least partly due to resistance to CYP24A1 [328]. Alternatively, 24-hydroxylation of the analog KH1060 has been implicated as one of the mechanisms to explain the potency of this analog. The 24-hydroxylated metabolites of this analog are very stable and remain biologically active [554,555]. It has been shown that the naturally occurring 24-hydroxylated metabolite of vitamin D ( $24\text{R},25-(\text{OH})_2\text{D}_3$ ) also has a preventive effect on chemically induced colon cancer [556].

In contrast to degradation of  $1,25(\text{OH})_2\text{D}_3$  by CYP24A1 in cancer cells, recently it has become clear that tumor cells contain CYP27B1 activity and thereby are able to locally generate  $1,25(\text{OH})_2\text{D}_3$ . Expression of  $1\alpha$ -hydroxylase has been demonstrated in colorectal cancer [557]. It was postulated that in early stages, tumor cells respond by upregulating  $1\alpha$ -hydroxylase activity to counteract neoplastic growth, while at later stages of tumor development, this is lost [557]. Also in prostate

cancer [558] and inflammatory myofibroblastic tumor [559], CYP27B1 has been detected, albeit that in the latter case, the tumor contains large numbers of macrophages. Intratumoral production of  $1,25(\text{OH})_2\text{D}_3$  by CYP27B1 delays breast cancer progression in a mouse model [560]. Over the years, it has become clear that VDR but also CYP27B1, CYP24A1, and vitamin D metabolism in the tumor (cancer cells and tumor stroma including immune cells) are important determinants of the vitamin D anticancer effects. An overview of the signaling pathways of vitamin D in cancer and their role in therapeutic involvement is shown by Deep et al. [561]; a review of molecular mechanisms underlying the positive effects of vitamin D in cancer is given by Fleet et al. [562].

HL60 cells that have been cultured for 4 years in the presence of  $1,25(\text{OH})_2\text{D}_3$  became vitamin D resistant. This resulted in clones that are resistant to differentiation induction and growth inhibition. They became not only resistant to vitamin D but also to 5-beta-D-arabino-cytosine suggesting a common metabolic pathway being responsible [563]. Whether this relates to the upregulation of the multidrug resistance proteins is not clear. Development of therapy resistance is a common phenomenon. Recurrent tumors are often resistant to therapy. Adding to the complexity of this phenomenon is the presence of a specific subset of cancer cells: the cancer stem cells. These cells are highly resistant to therapies and effective in repopulating the tumor [564]. Mammospheres isolated from breast cancer cell lines showed suppressed VDR signaling, but combined treatment with  $1,25(\text{OH})_2\text{D}_3$  and a nitric oxide (NO)-donor caused a significant decrease in mammosphere size and smaller tumor volume in nude mice [565]. Inhibition of breast cancer stem cell spheroid formation by  $1,25(\text{OH})_2\text{D}_3$  was also found by Jeong et al. [566]. Effects of vitamin D on prostate progenitor/stem cells resulted in cell cycle arrest, senescence, and differentiation, which was mediated by IL-1 $\alpha$  [567]. These strategies may lead the way to find new concepts to overcome therapy resistance. Targeting cancer stem cells by vitamin D is reviewed by So and Suh [568]. Currently, numerous studies focus on the effect of vitamin D on cancer stem cells or stem-like cells. The resistance to therapeutic drugs is due to the expression of multidrug-resistant proteins on the cell membrane. ATP-dependent transporters (ATP-binding cassette (ABC) transporters) can make cancer cells multidrug resistant [569,570]. Reversal of cancer drug resistance is a potential target for vitamin D [571,572]. As the ABC transporters require energy (ATP) to transport drugs, it is conceivable that vitamin D may affect this process as it has been shown that vitamin D regulates energy metabolism in cancer cells [380]. However, yet there are limited studies on vitamin D and multidrug resistance. A study showed vitamin D effects alone and in

combination with curcumin on multidrug resistance protein 1 (ABCB1) expression and paclitaxel sensitivity in MCF-7 breast cancer cells [573]. Further studies are absolutely required to investigate vitamin D and multidrug resistance.

## 6. Stimulation of proliferation

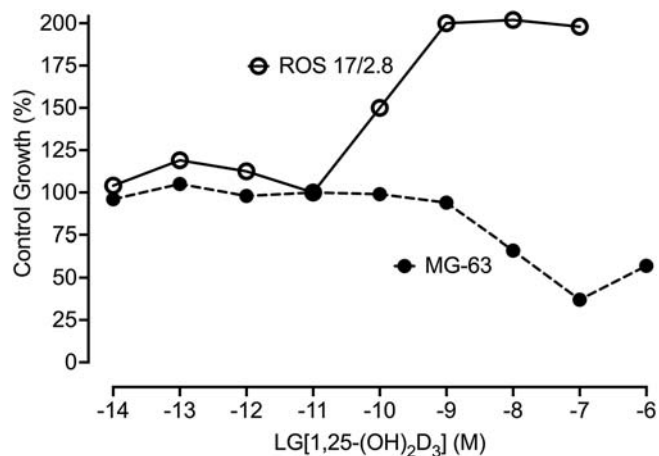
Over the years, a limited number of studies have demonstrated that, in contrast to growth inhibition,  $1,25(\text{OH})_2\text{D}_3$  can also stimulate tumor cell growth and tumor development. In several cells,  $1,25(\text{OH})_2\text{D}_3$  has been reported to have a biphasic effect, that is, at lower concentrations ( $<10^{-9}$  M), it stimulates proliferation, and at higher concentrations ( $10^{-9}$  to  $10^{-7}$  M), it inhibits proliferation. However, growth stimulation can sometimes be observed not only at low concentrations but also at the concentrations generally found to inhibit tumor cell proliferation and tumor development.  $1,25(\text{OH})_2\text{D}_3$  has been shown to stimulate the growth of a human medullary thyroid carcinoma cell line [574]. Not only cancer cells but also several normal cells, for example, human monocytes [575], smooth muscle cells [576], endothelial cells [506], and alveolar type II cells [577], are stimulated to grow by  $1,25(\text{OH})_2\text{D}_3$ . Skin is another organ in which different effects of  $1,25(\text{OH})_2\text{D}_3$  have been observed. In vivo studies demonstrated that  $1,25(\text{OH})_2\text{D}_3$  and analogs stimulate keratinocyte proliferation in normal mice [578–581] and enhance anchorage-independent growth of preneoplastic epidermal cells [582]. In contrast, other studies showed  $1,25(\text{OH})_2\text{D}_3$  inhibition of proliferation of mouse and human keratinocytes [583,584], and  $1,25(\text{OH})_2\text{D}_3$  is also effective in the treatment of the hyperproliferative disorder psoriasis [585]. Moreover, in vivo studies demonstrated that, depending on the carcinogen,  $1,25(\text{OH})_2\text{D}_3$  can either reduce [92] or enhance the induction and development of skin tumors in mice [586,587]. In addition,  $1,25(\text{OH})_2\text{D}_3$  enhances the chemically induced transformation of BALB 3T3 cells and hamster embryo cells [588,589].  $1,25(\text{OH})_2\text{D}_3$  also enhanced 12-O-tetradecanoylphorbol-13-acetate-induced tumorigenic transformation of mouse epidermal JB6 Cl41.5a cells [590,591]. Recently, in the model of the 4T1 mouse mammary gland cancer transplanted subcutaneously, Cao et al. have shown that vitamin D stimulated the growth of primary tumors and decreased survival time of mice. The authors correlated this unfavorable effect with decreased Th1 response and increased recruitment of myeloid-derived suppressor cells [592]. A complex relation between vitamin D and proliferation was observed in colorectal cancer cells and squalene epoxidase (SQLE), one of the rate-limiting steps in cholesterol biosynthesis.



SQLE stimulates cancer cell proliferation via a combination of  $1,25(\text{OH})_2\text{D}_3$  accumulation and increased CYP24A1 expression. The authors conclude that  $1,25(\text{OH})_2\text{D}_3$  is more essential than cholesterol in stimulating cancer cell growth [593]. Although further studies are needed to fully grasp this observation by He et al. [380] it further links  $1,25(\text{OH})_2\text{D}_3$  to cancer cell energy metabolism.

Another example on stimulation of proliferation comes from research on osteosarcoma cells. In 1986, it was shown that  $1,25(\text{OH})_2\text{D}_3$  stimulated the growth of tumors in athymic mice inoculated with the ROS 17/2.8 osteosarcoma cell line [594]. Earlier the same group reported growth stimulation in vitro of these osteosarcoma cells at low concentrations but growth inhibition by  $10^{-8}$  M [515]. They speculated that this discrepancy resulted from limited in vivo availability of  $1,25(\text{OH})_2\text{D}_3$  for the tumor cells, resulting in concentrations shown to be growth stimulatory in vitro. However, in other experiments with nude mice, the availability of  $1,25(\text{OH})_2\text{D}_3$  did not seem to be a factor, as growth inhibition was observed (see Table 84.2). In particular, in nude mice implanted with human osteosarcoma cells (MG-63), growth inhibition and tumor suppression by  $1,25(\text{OH})_2\text{D}_3$  were observed [102]. In two different in vitro studies, growth inhibition of MG-63 and growth stimulation of ROS 17/2.8 cells was reported [595,596]. For smooth muscle cells, it has been demonstrated, for example, that growth inhibition or stimulation can depend on the presence of additional growth factors in the culture medium [576]. We followed up on this concept by comparing the effects of  $1,25(\text{OH})_2\text{D}_3$  and analogs on the growth and osteoblastic characteristics of the two osteosarcoma cell lines under identical culture conditions. At concentrations  $10^{-10}$  to  $10^{-7}$  M  $1,25(\text{OH})_2\text{D}_3$  caused an increase in cell proliferation by 100% in ROS 17/2.8 cells, whereas the proliferation of MG-63 cells was inhibited (Fig. 84.2) [249]. In contrast, in both cell lines,  $1,25(\text{OH})_2\text{D}_3$  stimulated osteoblast differentiation characteristics such as alkaline phosphatase activity and production of osteocalcin [249,595]. Analyses with another steroid hormone demonstrated that glucocorticoids inhibited the growth of both osteosarcoma cell lines [597,598]. These data indicate specific differences between these cell lines, especially with respect to the  $1,25(\text{OH})_2\text{D}_3$  growth regulatory mechanisms.

In addition to these biological data in cells, an epidemiological study also showed an increased risk of aggressive prostate cancer with higher levels of 25-hydroxyvitamin D<sub>3</sub> [61]. A metaanalysis by Xu et al. [599] reported a positive association between high levels of 25(OH)D<sub>3</sub> and prostate cancer. Although the authors mention several justified limitations of their study, together with the data on growth stimulation and tumor development (although detected in only a small



**FIGURE 84.2** Effect of  $1,25(\text{OH})_2\text{D}_3$  on proliferation of the osteosarcoma cell lines ROS 17/2.8 and MG-63. Effects on proliferation were examined as described by van den Bemd et al. [249].

minority of cancer cells), it demonstrates that treatment with  $1,25(\text{OH})_2\text{D}_3$  or analogs may not always cause growth inhibition and tumor size reduction. It is therefore of utmost importance to identify the mechanism(s) by which  $1,25(\text{OH})_2\text{D}_3$  exerts its inhibitory and stimulatory effects on cell growth and what the contextual conditions are when  $1,25(\text{OH})_2\text{D}_3$  stimulates cancer cell growth. This may provide tools to assess whether treatment of a particular tumor will be beneficial. The observations on growth inhibition and stimulation may also be related to tumor heterogeneity.

## 7. Conclusions

The data obtained so far, on (1) the distribution of the VDR in a broad range of tumors and (2) the inhibition of cancer cell growth, angiogenesis, metastasis, inflammation, and PTHrP synthesis as well as the stimulation of differentiation and apoptosis by  $1,25(\text{OH})_2\text{D}_3$  all hold promise for the development of treatment strategies based on vitamin D use in a wide range of cancers. Moreover, combination of vitamin D with other anti-tumor drugs, hormones, or growth factors is an important additional therapeutic option. Throughout the previous decade, data have accumulated on the cellular targets and mechanisms of action of  $1,25(\text{OH})_2\text{D}_3$ -induced cancer growth inhibition. The clinical application is enhanced by the development of  $1,25(\text{OH})_2\text{D}_3$  analogs with potent growth inhibitory actions and reduced hypercalcemic activity. Nevertheless, it is crucial for the coming years to deliver strong clinical trials to support the potential of vitamin D in cancer treatment uncovered by investigation of cultured cells, animal models, and epidemiological studies. In the

meantime, continuing research to understand the mechanisms by which vitamin D<sub>3</sub> exerts its effects on tumor cell growth is needed so that therapeutic modalities may be employed more effectively.

Finally, yet most data on vitamin D and effects on cancer cells are described based on a broad variety of tumor cell models. These are mainly described on basis of origin of the tumor, e.g., breast, prostate, lung, etc. Overall, vitamin D effects on general processes such as proliferation, differentiation, and cell death/apoptosis are described with a broad variety and some contradictory effects at the molecular level regarding impact on or involvement of specific genes. To take a next step in the molecular studies on vitamin D mechanism of action in cancer efforts should be made to classify the cellular/tumor models on basis of their underlying mutational profile, process of tumorigenesis, and the clinical phenotype. Studying the effect(s) of vitamin D against the backdrop of this knowledge will provide more robust insights into the vitamin D action in cancer and may improve the design of clinical studies.

## 8. Summary points

- There is a wealth of preclinical data demonstrating inhibition of tumor cell growth by vitamin D, vitamin D metabolites, and a range of vitamin D analogs supporting epidemiological association studies.
- Vitamin D impacts a broad range of well-known molecular targets in cancer cell biology, cell proliferation, and cell death, thereby providing a mechanistic basis for the observed cancer cell growth effects.
- Clinical studies are not unambiguously providing evidence for a therapeutic role of vitamin D in cancer.
- Vitamin D may act as an accelerator/facilitator of other anticancer drugs (combination therapies) and/or have a more preventive role (DNA repair, apoptosis, autophagy, etc.)
- Intratumor vitamin D metabolism and heterogeneity as well as multidrug resistance mechanisms may hamper the vitamin D effect, but are also potential targets to improve the clinical outcome.

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# Vitamin D status and cancer incidence, mortality, and prognosis

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## OBJECTIVES

- Characterize the main types of population-based studies contributing to our knowledge of vitamin D and cancer incidence and progression.
- Summarize the main findings of the cohort studies examining 25-hydroxyvitamin D and cancers of the colorectum, breast, prostate, lung, and other cancers.
- Summarize the main findings of the randomized controlled trials of vitamin D and total cancer incidence.
- Summarize the main findings of the ecologic studies, which determine average vitamin D status in the population, on cancer rates.
- Summarize the findings on vitamin D status and cancer mortality, both in studies among healthy populations and cancer patients (survival).
- Summarize the main findings of the randomized controlled trials of vitamin D and total cancer incidence.
- Summarize the main findings of studies using genetic approaches, including Mendelian randomization, to examine genetic variation of vitamin D status or variation in the vitamin D receptor on cancer incidence and mortality.
- Describe the main challenges inherent in all epidemiologic studies of vitamin D and cancer.

## 1. Introduction

In the 1930s, Peller and Stephenson observed that US Navy personnel had higher rates of skin cancer, but lower rates of other cancers [1]. From this observation, they hypothesized that exposure to sunlight induced skin cancer, which then conferred immunity against other cancers. Several years later, Apperly observed an association between latitude and cancer mortality rate which he attributed to direct effects from exposure to solar radiation [2]. These observations were essentially ignored by the scientific community for about four decades. In the early 1980s, Garland and Garland hypothesized that the potential benefit of sun exposure was attributed to vitamin D, which by then was known to be made from solar ultraviolet B (UV-B) radiation [3]. At the time, there was no mechanism proposed why vitamin D would have anticancer effects. Yet in subsequent years, laboratory studies supported several anticancer properties of vitamin D, including reduced proliferation, invasiveness, angiogenesis, and metastatic potential and increased differentiation and apoptosis [4,5]. These are detailed in other chapters of this section of the book. A growing number of studies have focused on investigating the relationship between vitamin D and cancer prognosis and survival. Indeed, it is plausible that vitamin D could affect tumor biologic aspects of aggressive behavior, such as advanced stage, poor differentiation, metastasis, and survival.

This chapter provides a review and synthesis of epidemiologic evidence focused on the relationship between vitamin D and cancer, and identifies gaps in this evidence where future research is needed. Plausibly, vitamin D could affect either or both cancer incidence (occurrence) or cancer mortality. When considering cancer mortality, it is important to consider that the relevant timing of the exposure on outcome could be during the prediagnostic or postdiagnostic period. In addition, when cancer mortality is an outcome for a cohort that was cancer-free at baseline, cancer mortality may be influenced by either incidence or prognosis. For example, if a risk factor doubled the incidence of a cancer, and the cancers attributed by the risk factor were equally aggressive as those not related to the exposure, we would anticipate a doubling of cancer mortality (assuming no large effect of competing causes). If the action is primarily on survival, the effects can occur either during the prediagnostic period or postdiagnostic period, or both. In this chapter, we first discuss studies that examine vitamin D, and specifically vitamin D deficiency, in relation to incidence, and then on mortality and survival.

## 2. Cancer incidence

Ecologic studies examine rates of cancer incidence or mortality in an entire population, rather than on an individual basis, and then correlate some surrogate of vitamin D to the cancer rates across populations (see Chapter 58). Many of the ecologic studies that initiated the vitamin D–cancer hypothesis were based on cancer mortality in the population. Thus, these studies did not clearly distinguish if any effect, if causal, is primarily due to incidence or mortality. Nonetheless, these studies are compatible with the hypothesis that improved vitamin D status may reduce risk of cancer, and vitamin D deficiency increases risk, “particularly colorectal cancer.” Yet, as many factors other than sun exposure and hence vitamin D status can vary with latitude, which is the typical way vitamin D status is assessed in these studies, there are alternative causal explanations. In the recent two to three decades, there have been a number of reports of prospective cohort studies that have assessed circulating 25-hydroxyvitamin D (25(OH)D) levels in relation to risk of cancer incidence for various cancers using a prospective nested case–control design. In these studies, typically blood samples are taken in a population at baseline and archived, and individuals are followed for cancer risk. The prediagnostic 25(OH)D levels in the subsequent cancer cases and matched controls are assessed and are compared. As these prospective studies, based on a direct measurement of vitamin D status, are generally considered the strongest

design for observational studies of vitamin D (see [Chapter 53](#)), we review primarily these studies here, though other studies, such as those on dietary intake, are also mentioned when relevant.

### 2.1 Colorectal cancer or adenoma

Colorectal cancer was the first malignancy hypothesized to be associated with vitamin D deficiency and has been relatively well studied. Studies that have examined circulating 25(OH)D levels prospectively in relation to risk of colorectal cancer have generally supported an inverse association [6–14]. In an initial metaanalysis of the colorectal cancer studies, based on 535 cases, individuals with  $\geq 82$  nmol/L (33 ng/mL) serum 25(OH)D level had 50% lower incidence of colorectal cancer ( $P < .01$ ) compared with those with vitamin D deficient concentrations at levels of less than or equal to 30 nmol/L (12 ng/mL) [15]. The dose–response appeared to be linear up to a 25(OH)D concentration of at least approximately 90 nmol/L (36 ng/mL), with no obvious threshold or nonlinear relationship. Controlling for multiple covariates, including physical activity, body mass index, and various dietary factors, has had little influence on the findings. These results were confirmed in a metaanalysis of 2630 colorectal cancer cases based on the literature up to December 2009 [16]. In that study, the summary relative risk (RR) was 0.85 (95% confidence interval [CI] = 0.79–0.91) for a 25 nmol/L increment in 25(OH)D. That is, risk decreased by 15% for every 25 nmol/L higher 25(OH)D level.

Many of the existing large cohort studies with plasma or serum biomarkers have published on this association, and most found an inverse association as reflected in the metaanalysis [16]. The large studies that have supported this association have come from diverse populations. These include the Nurses’ Health Study [8], the Health Professionals Follow-Up Study [17], the Women’s Health Initiative [14], the Japan Public Health Center-based Prospective Study [18], the European Prospective Investigation into Cancer and Nutrition study (EPIC), which is a cohort of more than 520,000 participants from 10 western European countries [19], and the Multiethnic Cohort including men and women of Japanese, Latino, African American, White, and Native Hawaiian ancestry [20]. In none of these studies, which had extensive information on various diet and lifestyle factors, was any confounding factor found to completely account for the association. The conclusion was that vitamin D deficiency was associated with increased colon cancer incidence, and there was no evidence of an obvious confounding factor.

Several studies have examined circulating 25(OH)D concentrations and the risk of colorectal adenomas. Adenomas (especially large and high-grade) are well

established precursors to colorectal cancer, and most colorectal cancers are thought to arise from adenomas. Because adenomas are largely asymptomatic, most of the studies compared 25(OH)D levels in adenomas cases to controls who were adenoma-free on colonoscopy or sigmoidoscopy. The studies were based either on initially diagnosed adenomas in the participants, or in adenomas among individuals who had had an adenoma and were then followed for subsequent or recurrent adenomas. In general, these studies suggest either a statistically significant or a nonsignificant inverse association with 25(OH)D and possibly 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) [9–12,21], particularly for advanced adenomas [12]. In a metaanalysis that summarized the results for all available studies of circulating 25(OH)D and adenoma risk [22], higher circulating 25(OH)D was associated with lower risk of colorectal adenomas; the  $\text{RR} = 0.70$  (95%  $\text{CI} = 0.56\text{--}0.87$ ) for high versus low circulating 25(OH)D. The inverse association was stronger for advanced adenoma ( $\text{RR} = 0.64$ ; 95%  $\text{CI} = 0.45\text{--}0.90$ ), though only a fraction of the studies reported on advanced adenomas. In general, the studies of adenomas tend to support an association between vitamin D deficiency and higher risk of colorectal neoplasia.

In another recent metaanalysis, higher circulating 25(OH)D was inversely associated with risk of colorectal adenoma ( $\text{RR}: 0.80$ , 95%  $\text{CI}: 0.71\text{--}0.89$ ). Vitamin D intake was also associated with risk of adenoma ( $\text{RR}: 0.87$ , 95%  $\text{CI}: 0.82\text{--}0.92$  [23]). In addition, colorectal cancer incidence was inversely associated with circulating 25(OH)D ( $\text{RR}: 0.67$ , 95%  $\text{CI}: 0.59\text{--}0.77$ ) and vitamin D intake ( $\text{RR}: 0.85$ , 95%  $\text{CI}: 0.78\text{--}0.93$ ), and higher circulating 25(OH)D was associated with better overall survival ( $\text{RR}: 0.67$ , 95%  $\text{CI}: 0.57\text{--}0.79$ ) and colorectal-specific survival ( $\text{RR}: 0.63$ , 95%  $\text{CI}: 0.53\text{--}0.74$ ). These data suggested that vitamin D may act at various stages of colorectal neoplasia. Notably, the associations tended to be stronger in European and American populations compared with Asian populations, and in populations with higher calcium intake. Asian populations tend to have lower intake of calcium, which could potentially account for the weaker associations with vitamin D.

One study pooled participant-level data of circulating 25(OH)D concentrations from 17 cohorts [24]. This pooled study comprised 5706 colorectal cancer case participants and 7107 control participants. For 30% of participants, 25(OH)D was newly measured, and previously measured 25(OH)D was calibrated to the same assay to permit estimating risk by absolute concentrations. This approach of pooling individual level data gives an advantage over the more typical metaanalyses, which combine data from studies using different assays. In this study, compared with 25(OH)D levels of  $50\text{--}<62.5$  nmol/L, deficient 25(OH)D levels ( $<30$  nmol/L)

were associated with 31% higher colorectal cancer risk ( $\text{RR} = 1.31$ , 95%  $\text{CI} = 1.05\text{--}1.62$ ); 25(OH)D levels of  $75\text{--}<87.5$  nmol/L were associated with 19% lower risk ( $\text{RR} = 0.81$ , 95%  $\text{CI} = 0.67\text{--}0.99$ ); and levels of  $87.5\text{--}<100$  nmol/L were associated with 27% ( $\text{RR} = 0.73$ , 95%  $\text{CI} = 0.59\text{--}0.91$ ) lower risk. The inverse association was stronger in women compared with men.

In various populations, including the United States, colorectal cancer rates have been increasing in those under age 50 in recent decades. It is unknown if vitamin D status is related to this change in rates. One recent study of women in the United States suggested that low vitamin D intake is associated with higher risk of colorectal cancer and adenomas in individuals under the age of 50 [25]. This finding needs replication.

## 2.2 Breast cancer

Breast cancer is one of the cancers that has been hypothesized to be associated with vitamin D. However, the epidemiologic data based on circulating 25(OH)D concentrations have not been consistent, and overall, are not supportive of an association. A metaanalysis in 2011 found different results for circulating 25(OH)D and breast cancer risk based on study design [16]. For prospective studies, where the blood sample was collected prior to the occurrence of breast cancer, no significant association was found. For a 25 nmol/L increment in 25(OH)D, the summary  $\text{RR}$  in the metaanalysis was 0.97 (95%  $\text{CI} = 0.92\text{--}1.03$ ) based on 3145 cases. However, for case–control (retrospective studies), where data were collected after the diagnosis, an inverse association was found (corresponding  $\text{RR} = 0.83$ ; 95%  $\text{CI} = 0.79\text{--}0.87$ ) based on 3030 cases. For the case–control studies, a number of issues were noted, including assessing blood status long after the diagnosis allowing more potential for changes in vitamin D status that do not reflect prediagnostic values, and low participation rates among controls allowing for potential selection bias. Retrospective case–control studies of 25(OH)D and cancer are prone to biases, and the divergent results from prospective and retrospective studies for breast cancer may well reflect these biases.

Similar conclusions were observed in a metaanalysis of prospective studies conducted in 2014 [26]. This metaanalysis included 30 prospective studies and reported a summary  $\text{RR}$  of 0.92 (95%  $\text{CI}: 0.83\text{--}1.0$ ). While not statistically significant, this study was supportive of a modest 8% reduction in risk. No heterogeneity was observed for menopausal status. In another metaanalysis conducted in 2019, an inverse association between 25(OH)D level and breast cancer risk was observed in case–control studies (for high vs. low comparison ( $\text{RR} = 0.57$ , 95%  $\text{CI}, 0.48\text{--}0.66$ ), but not in cohort studies ( $\text{RR} = 1.17$ ,



95% CI, 0.92–1.48) [27]). This metaanalysis also showed no significant association between vitamin D intake and breast cancer risk.

Thus, based on studies of circulating 25(OH)D, no clear association has been observed between 25(OH)D level and breast cancer incidence, at least in studies where the specimen is collected before diagnosis. The main limitation of these studies is that only a single measure of 25(OH)D concentration was made, mostly in middle age, which would tend to attenuate any associations apparent for long-term vitamin D status. The strong inverse association in case–control studies is in stark contrast to the prospective studies. The most likely explanation is reverse causation, that is, breast cancer diagnosis leads to poorer vitamin D status rather than poor vitamin D status leads to breast cancer. For breast cancer incidence, early-life exposure is a particularly important point for etiologic factors, and the studies do not address early-life stage effects.

### 2.3 Prostate cancer

Most of the studies of circulating 25(OH)D level and prostate cancer risk have not found clear risk reductions associated with higher 25(OH)D levels, although some of the studies suggested weak inverse associations [13,28–32]. Two studies [33,34] that tend to support an inverse association between 25(OH)D and prostate cancer risk were conducted in high-latitude Nordic countries, where 25(OH)D levels may be particularly low due to low solar UV-B exposure. However, even the findings from these studies were equivocal. In fact, one of these studies suggested a U-shaped relationship between vitamin D and prostate cancer risk. Large studies also found no association between higher 25(OH)D levels and lower risk for prostate cancer. In the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, an analysis based on 749 cases and 781 controls found no association, and even a suggestively increased risk of aggressive prostate cancer (Gleason sum  $\geq 7$  or clinical stage III or IV) among men with higher circulating 25(OH)D levels [35]. However, this population by nature of the study design is extensively screened with prostate-specific antigen (PSA) measurements, so the vast majority of the prostate cancers were not of advanced stage (e.g., metastatic), and aggressive prostate cancers were mostly high-grade but organ-confined cancers. In the EPIC study, serum 25(OH)D levels were measured in 652 prostate cancer cases matched to 752 controls from seven European countries [36]. The median follow-up time for the study was 4.1 years. No significant association was found between 25(OH)D concentration and risk of prostate cancer (RR for highest vs. lowest quintile = 1.28, 95% CI = 0.88–1.88; *P* trend = 0.188).

Subgroup analyses showed no significant heterogeneity by cancer stage or grade. In Europe, there is relatively low screening rate for prostate cancer by prostate-specific antigen, so these cases were likely of a relatively more advanced stage than in the studies based in the United States.

The lack of an association between circulating 25(OH)D and risk of prostate cancer was confirmed in a metaanalysis [16]. Based on 3956 cases in 11 identified studies, the summary RR for a 25 nmol/L increment in circulating 25(OH)D was 0.99 (95% CI = 0.95–1.03). Clearly, studies of circulating 25(OH)D concentration have tended not to support an association for prostate cancer, or at best, have yielded equivocal results, with suggestive results possibly at the very deficient low end of the 25(OH)D range (e.g.,  $< 20$  nmol/L). Null associations or even suggestive positive associations for total prostate cancer incidence have been confirmed in more recent metaanalyses [37–39]. The positive association seen in some studies is concerning; however, prostate cancer diagnosis is very prone to detection bias. For example, it is possible that more health-conscious men, who may have higher 25(OH)D levels, may be more likely to have PSA examinations and greater likelihood for detection of otherwise indolent and latent cancers. Populations with very low 25(OH)D concentrations and at notably higher risk of prostate cancer, particularly African Americans, have not received adequate study in regard to vitamin D.

### 2.4 Other cancers

Most cancers besides colorectal, breast, and prostate cancers are generally too uncommon to study with adequate power in individual cohorts. Thus, to address 25(OH)D deficiency in relation to risk of these less common cancers, the Cohort Consortium Vitamin D Pooling Project of Rarer Cancers (VDPP) was formed [40]. This consortium brought together 10 cohorts to conduct a prospective study of the association between concentrations of 25(OH)D and risk of seven less common cancer sites. These cohorts had stored plasma or serum samples and long-term follow-up for cancer. The malignancies studied in the VDPP included endometrial, esophageal, gastric, kidney, non-Hodgkin lymphoma, ovarian, and pancreatic cancers. For this consortium, seven of the cohorts were based in the United States, one in Finland and two in China. The participants were from a wide range of latitudes in various countries, potentially broadening the range of exposure to solar UV-B. The total numbers of cases for each of the cancer sites were as follows: endometrium 830, upper gastrointestinal (esophagus/stomach) 1,065, kidney 775, non-Hodgkin's lymphoma 1353, ovary 516, and pancreas

952. The consortium used a standard laboratory to examine 25(OH)D in a nested case–control sample in the various studies, and then pooled the results. The overall design, description of the cohorts, and statistical methodology were outlined in a methods paper [40]. For all studies, the reported circulating vitamin D categories were <25, 25–<37.5, 37.5–<50, 50–<75, 75–<100, and  $\geq 100$  nmol/L. In general, no overall association was observed in these studies, with no trends approaching statistical significance [40–46]. Some notable subgroup findings are discussed in the following.

For all the cancers, compared with the reference category of 50–75 nmol/L, no comparison group was statistically different, except for an increased risk of pancreatic cancer associated with concentrations of 25(OH)D greater than 100 nmol/L (adjusted odds ratio 2.12, 95% CI = 1.23–3.64). However, a subsequent pooled analysis of other studies in the United States did not confirm this finding and even suggested an inverse association between circulating 25(OH)D and pancreatic cancer risk [47]. For ovarian cancer, as for the other cancers, no overall association was observed, although there was statistically significant interaction with body mass index (BMI) ( $P < .01$ ), with an inverse association suggested only for women with a body mass index  $\geq 25$  kg/m<sup>2</sup> ( $P$  trend = 0.01). Two prospective nested case–control studies conducted in the Finnish Maternity Cohort provide evidence of an inverse association between 25(OH)D and ovarian cancer for a relatively young age-at-onset group of ovarian cancer. In the first report, the RR among women with insufficient (<75 nmol/L) serum 25(OH)D concentration was 2.7 (95% CI = 1.0–7.9) compared with that among those with sufficient (>75 nmol/L) serum 25(OH)D concentration [48]. In the second report, having sufficient compared with insufficient serum 25(OH)D levels was associated with a significantly decreased risk of ovarian cancer (RR 0.32; 95% CI = 0.12–0.91) [48].

In the VDPP, overall, no association was observed between 25(OH)D level and risk of upper gastrointestinal cancer [42]. However, some difference appeared by race. Among Caucasians, no overall trend was observed, but above levels of 25 nmol/L of 25(OH)D, a suggestive inverse trend was observed with increasing levels, though those with levels <25 nmol/L were not at higher risk. In contrast, among Asians, a significant positive trend was noted with higher circulating 25(OH)D ( $P$  trend = 0.003). However, in one of the two main Asian cohorts (Shanghai Men's Health Study), the median follow-up time was only 1.7 years, suggesting the possibility of reverse causation. Further suggestive of reverse causation, the association with 25(OH)D was stronger in follow-up less than 2 years than in follow-up time  $\geq 2$  years. This may suggest that latent cancers affected 25(OH)D levels.

## 2.5 Randomized controlled trial studies of vitamin D and total cancer incidence

In recent years, data on vitamin D supplementation and cancer incidence from randomized controlled trials (RCTs) have increased. In most studies, RCTs generally have not been conducted for cancer incidence as a primary outcome. The largest RCT, which did have cancer incidence as a primary endpoint, was the Vitamin D and Omega-3 Trial (VITAL). This was a large-scale randomized trial conducted in the United States in which over 20,000 participants were randomized to take either 2000 IU of vitamin D<sub>3</sub> (cholecalciferol) per day or a placebo over a 5-year period. The primary endpoints were total cancer and major cardiovascular events; site-specific cancers and cancer mortality were secondary endpoints. No association was observed for vitamin D versus placebo with cancer incidence. The hazards ratio (HR) was 0.96 (95% CI = 0.88–1.06) [49]. Another recent study was the D-Health Trial [50], a randomized placebo-controlled trial in Australia, with intervention for 5 years, and with 5 years of additional passive follow-up. 21,315 participants aged 65–84 years were recruited and given monthly oral dose of 60,000 IU of cholecalciferol or placebo. This study also showed no effect on cancer incidence; the hazard ratio was 1.01 (95% CI = 0.81–1.25).

The available studies have generally been underpowered to examine specific cancer subtypes, but had reasonable power to examine total cancer incidence. A recent metaanalysis considered the available RCTs of vitamin D supplementation and total cancer incidence for studies up to January 2022 [51]. For total cancer incidence (12 trials), the summary RR for vitamin D supplementation versus control group was 0.99 (95% CI, 0.94–1.03;  $P = .54$ ;  $I^2 = 0\%$ ). No significant association was indicated for any of the daily versus infrequent large-bolus dosing strategies ( $P_{\text{heterogeneity}} = 0.40$ ). The finding was statistically robust, as illustrated by the tight confidence intervals. A wide range of starting 25(OH)D levels were seen, with the mean/median levels of circulating 25(OH)D in the participants ranging from approximately 38–83 nmol/L at baseline. By dosing frequency, eight RCTs provided vitamin D<sub>3</sub> supplements daily (400–4000 IU/day), while five RCTs provided a large bolus dose infrequently (20,000 IU/week to 500,000 IU/year). The levels in the intervention groups elevated to 54–136 nmol/L at a point during the follow-up. The mean durations of the total follow-up periods, including intervention and postintervention follow-up, ranged approximately 3–10 years. An unexpected but potentially interesting result was when metaanalyses were conducted for each category of BMI, the results were significantly heterogenous across

BMI level ( $P_{\text{heterogeneity}} = 0.02$ ), and a significant inverse association observed only among normal weight individuals (RR, 0.76; 95% CI, 0.64–0.90;  $P = .001$ ), but not among overweight or obese individuals. Yet this result was largely driven by the VITAL study and thus requires confirmation.

Although considered the gold standard for evidence, randomized controlled trials (RCTs) generally have limitations for studying a complex association such as that for vitamin D and cancer risk. Adult cancers typically arise through a long multistage process. At what stage the exposure (i.e., vitamin D) is relevant and the required duration are not accounted for in studies that implicitly study relatively short durations of vitamin D status at late stages of the cancer occurrence. Furthermore, it is important to choose an appropriate dose, and to select a study population that is able to benefit from supplementation (e.g., one that is deficient in vitamin D). Even if the study design is appropriate, there may be practical issues such as ensuring compliance and avoiding contamination (e.g., those assigned to the placebo begin taking vitamin D supplements) that can undermine the study. If the conditions for the study design, power, and execution are not met, a real association could be attenuated and even missed. Thus, while RCTs could provide the strongest evidence for a causal association, RCTs with null results should be considered in the context of other lines of evidence and may not necessarily be the final word. It is also relevant that the metaanalysis was able to only consider total cancer incidence. Whether there is benefit for specific types of cancer remains an open question.

### 3. Cancer mortality and survival

The number of individuals with cancer worldwide is very large and increasing. For example, currently, in the United States (US) alone, there are more the 15 million cancer survivors, a number that is projected to grow to more than 20 million by 2026 [52]. Additionally, there is a high prevalence of vitamin D deficiency among those diagnosed with cancer; one review of 37 studies found that up to 67% of patients with cancer had vitamin D deficiency (levels less than 20 ng/mL or 50 nmol/L) [53]. Thus, if there is a causal relationship between vitamin D status and reduced cancer survival and prognosis, intervening on the population at large or among cancer survivors could have a large public health impact. In addition, although vitamin D does not appear to have a very strong association with total cancer incidence, other than perhaps colorectal cancer, it is plausible that it may affect cancer mortality by reducing the aggressiveness (e.g., metastatic potential) of cancers that occur.

### 3.1 Sun exposure

Latitude or region UV-B radiation has been examined in relation to cancer mortality in several ecologic studies. In general, ecologic studies have found that lower mortality rates of numerous types of cancer are correlated with regions with greater solar UV-B exposure [54–63]. The geographical association between UV-B exposure and cancer was stronger for cancer mortality as the endpoint than for incidence for many cancers in the United States and China [59,60]. An important limitation of these ecologic studies is that potential confounding factors related to regional differences in solar UV-B radiation could account for the associations. However, suggestive evidence that an inverse association between regional solar UV-B exposure and cancer mortality may be causal is that this association is observed in regions outside of the United States, including in Japan (for digestive organ cancers) [58], Spain [62], and China [59]. Thus, a putative confounding factor would have to have similar relationships with regional solar UV-B exposure in these diverse populations. A strength of ecologic studies is that they may provide some indication of long-term sun exposure, including during childhood and adolescence, thus preceding the onset of cancer by many years. Ecologic studies are discussed in more detail in [Chapter 53](#).

Unlike ecologic data, case–control and cohort studies assess exposure and outcome at the individual level. Confounding may be better controlled because typically more detailed information can be assessed on other covariates. Several case–control and cohort studies have assessed sun exposure in relation to cancer mortality. Freedman et al. [64] conducted a large US death certificate–based case–control study of cancer mortality to examine associations with residential and occupational exposure to sunlight. Residential sun exposure was inversely associated with mortality from breast, ovarian, prostate, and colon cancers. Mortality from nonmelanoma skin cancer, acting as a “positive control,” was positively associated with residential sun exposure. An analysis of NHANES I data based on 59 fatal prostate cancer cases among 3426 non-Hispanic white men found that frequent recreational sun exposure in adulthood was associated with a lower risk of fatal prostate cancer (RR = 0.47), and men who were both born in and had the longest residence in a high solar radiation region also had a lower risk of fatal prostate cancer (RR = 0.34) [65]. Conversely, a large prospective US study of almost 350,000 non-Hispanic white participants found that higher exposure to UV-B at baseline residence was associated with a small increased risk when comparing quartile 4 to quartile 1 (RR = 1.04, 95% CI = 1.02, 1.05) of overall cancer mortality in men. The main driver of this association was deaths from lung



cancer. While this study had large numbers and the ability to control for several confounding factors, UV-B exposure was only considered at baseline when participants were between 50 and 71 years of age and therefore may not reflect earlier-life exposures due to population mobility [66].

Region of residence as a surrogate of solar UV-B radiation and vitamin D status may be affected by increasing urbanization, more time spent indoors, winter vacations to sunny climates, and sun avoidance or sunscreen use. These factors could vary among populations and change over time. Some studies have used factors such as history of sunburns or holidays in a hot climate as proxies for sun exposure, and the findings have been mixed. A Swedish prospective cohort study of almost 40,000 women aged 30–49 years followed for a mean of 14.9 years found a protective association with all-cause mortality, but did not find an association between history of sunburns or going on sunbathing vacations between age 10 and 39 years and cancer-specific mortality. Artificial UV exposure was associated with increased all-cause and cancer-specific mortality [67]. An Italian study of over 2000 melanoma patients found that holidays with sun exposure were associated with favorable prognostic factors [68]. An international population-based study among 3578 cases of melanoma with a mean 7.4 years of follow-up found that having at least one sunburn within 10 year of diagnosis was associated with increased survival, but other measures of sun exposure, including water activities, skin damage, sunny holidays, and estimated ambient UV-B dose based on residence, were not associated with survival [69].

Season of diagnosis has also been used as a proxy for vitamin D status. Because season of diagnosis represents only a small fraction of the time of cancer development, for it to be relevant would require some acute benefit, such as an interaction with treatment shortly following diagnosis. Some studies found that patients diagnosed with cancer and/or treated during the summer and autumn months, when vitamin D status is higher, have a better prognosis than those diagnosed and/or treated during the winter months [70–76], while others did not [77,78]. While season of diagnosis may be correlated with circulating vitamin D levels, other factors could also confound the association. For example, a study based in Sweden did not find this pattern, but an explanation could be that the structure of the healthcare system and vacationing patterns might lead to disease diagnosed at a later stage in the summer [78].

### 3.2 Circulating 25(OH)D

The circulating 25(OH)D concentration is generally considered the best measure for total vitamin D status.

Serum 25(OH)D has a relatively long half-life of about 2–3 weeks and can provide a reasonable indicator of long-term (5–15 years) vitamin D status (see [Chapter 53](#)). The majority of studies to date that have assessed circulating 25(OH)D and cancer survival have used measures of postdiagnostic 25(OH)D. The time of blood collection varies in these studies from very close to diagnosis and prior to treatment to over a longer period after diagnosis. Only a few studies have collected prediagnostic blood samples, and the availability of multiple longitudinal samples is generally not feasible for large cohort studies. In studies in which 25(OH)D is measured after diagnosis in cancer patients, potential reverse causation needs to be considered, due to potential behavior/lifestyle changes following diagnosis or the disease itself affecting 25(OH)D levels. There is some evidence that the prevalence of 25(OH)D insufficiency is higher in cancer patients compared with the general population [79]. In particular, for blood collected at longer periods postdiagnosis, patients with more advanced disease with a worse prognosis may have lower vitamin D levels (for example, due to less sun exposure or physical activity). Studies in which the results were stronger when limiting the analyses to early stage cases or in studies that only examined early stage cases may be less susceptible to this possibility. It is also possible that if blood samples are collected posttreatment, that treatment could modify 25(OH)D levels. In studies that assess 25(OH)D in the prediagnostic period, reverse causation is less likely, but without multiple blood measurements, we cannot definitively determine the most relevant timing of the association, as prediagnostic and postdiagnostic 25(OH)D levels are likely to be correlated. Results of studies of circulating 25(OH)D concentration and cancer mortality, focusing on colorectal, breast, prostate, lung, and melanoma cancers, are briefly reviewed here.

### 3.3 Colorectal cancer

There is relatively consistent evidence for a protective association of circulating 25(OH)D concentrations and cancer prognosis with colorectal cancer. Several metaanalyses and reviews have found consistent evidence supporting a benefit of higher circulating 25(OH)D and survival and prognosis [79–83]. Some studies measured 25(OH)D from blood collected postdiagnosis [81,84,85], and others collected blood prediagnosis [86–88]. Zgaga et al. [81], in a prospective study of 1598 stage 1 to III colorectal cancer patients who experienced 363 CRC-specific deaths over a median of 9 years of follow-up, found that circulating 25(OH)D sampled postoperatively was inversely associated with colorectal cancer-specific mortality; the HR for the highest versus



lowest tertile = 0.68 (95% CI: 0.50–0.90) after multivariable adjustment. When they combined their results in a metaanalysis of four other published studies, they found the adjusted HR was 0.67 (95% CI, 0.54–0.81). Some studies have found that results varied by stage; one study found stronger association in stage II cancers [81], while another found stronger results in more advanced stage colorectal cancers [88]. Two studies specifically assessed advanced-stage disease and one found a protective association [89], while the other found no association [90].

A metaanalysis that examined circulating 25(OH)D in relation to colorectal cancer outcomes was conducted in 2018 [83]. This analysis included 11 original studies with a total of 7718 colorectal cancer patients. When comparing highest versus lowest categories of 25(OH)D, the pooled HR and 95% CI were 0.68 (0.55–0.85) and 0.67 (0.57–0.78) for overall and colorectal cancer-specific survival, respectively. A dose–response relation was observed. This recent metaanalysis provided strong evidence of an association between higher blood 25(OH)D concentrations and better overall and cancer-specific survival in colorectal cancer patients.

### 3.4 Breast cancer

Similar to colorectal cancer, several recent reviews and metaanalyses have found evidence favoring a protective association of higher circulating 25(OH)D with breast cancer prognosis and survival [79,80,82,91–93]. A metaanalysis in 2014 [92] included eight independent studies and reported low circulating 25(OH)D was associated with increased overall mortality ( $RR_{\text{lowest vs highest quartile}} = 1.52$ ; 95% CI: 1.22–1.88) and increased breast cancer-specific mortality ( $RR = 1.74$ ; 95% CI: 1.23, 2.40). These results have been largely confirmed in more recent metaanalyses [94,95].

Most studies have been conducted in early-stage (stage I–IIIa) patients. One study that examined both early- and late-stage patients found that the association was limited to those diagnosed in the early stage [92], which could be due to more advanced cancers being less amenable to lifestyle factors. The protective association may be stronger in studies with blood collected closer in time to diagnosis as two studies with longer times from diagnosis to blood collection did not show an association [91]. One potential explanation could be that circulating 25(OH)D levels may change due to therapy or changes in lifestyle in response to the cancer diagnosis or worsening disease [96]. For example, one study found that the association of circulating 25(OH)D and mortality in breast cancer patients was only statistically significant in those who did not receive chemotherapy or whose blood was collected prior to

chemotherapy [97]. However, three other studies in women with early-stage breast cancer participating in different breast cancer treatment trials have not observed associations between circulating 25(OH)D level and breast cancer prognosis [98–100].

Explanations for the differences between the results of the nontherapeutic observational studies and the results of these trials could be due the more restrictive study entry criteria for the trials resulting in a more homogenous population, or the potential for the adjuvant treatments to negate the adverse impact of low circulating 25(OH)D. Additionally, no information was available on vitamin D supplementation after blood draw, and studies have shown this to be common in recently diagnosed breast cancer patients [101].

### 3.5 Prostate cancer

The literature on prostate cancer mortality and prognosis with respect to circulating 25(OH)D is relatively supportive of an inverse association but overall inconsistent. Three studies have assessed postdiagnostic circulating 25(OH)D level and prostate cancer mortality, with one finding a protective association [102], and two others finding no significant association [103,104]. One of the studies that did not find an association enrolled only men with stage IV disease and had a relatively short median follow-up of 31 months [104]. As discussed previously, it is possible that more advanced cancers may be less susceptible to the influence of vitamin D levels and modifiable factors in general. Studies assessing prediagnostic circulating 25(OH)D level and prostate cancer mortality are also mixed. A cohort study of Finnish male smokers did not find an association of 25(OH)D level with incident fatal prostate cancer [105]. A US-based cohort study of males found that those in the highest quartile of plasma 25(OH)D had a 57% reduction in the risk of lethal prostate cancer compared with those in the lowest quartile [106]; data from this study were combined with another male US cohort study and men in the lowest quartile of prediagnostic plasma 25(OH)D had a 60% higher risk of progression to prostate cancer death compared with those in the highest quartile [107]. However, a pooled analysis combining the three cohorts mentioned above and two additional cohorts with a total of 518 fatal prostate cancer cases and 2986 controls did not find a significant association [108].

Two published survival analyses found evidence for a protective association. A Swedish study with 943 cases of prostate cancer and 169 deaths due to prostate cancer over a median of 9.1 years found an approximately 50% lower risk of prostate cancer-specific mortality in those with vitamin D levels above

85 nmol/L compared with those with 68 nmol/L or lower [109]. Interestingly, the cohort study of Finnish male smokers, who did not find an association of 25(OH)D with incidence of fatal prostate cancer compared with controls, did observe a statistically significant ~30% reduction of progression to prostate cancer–specific death among men diagnosed with prostate cancer who had the highest quintile of pre-diagnostic serum 25(OH)D compared with the lowest quintile [110]. The authors posited that the more recent survival analysis had longer follow-up and more end-points and thus may have been more sufficiently powered to detect an association. Another difference was that the prior analysis assessed the endpoint of fatal prostate cancer incidence compared with controls, whereas the later analysis assessed survival after diagnosis among cases only.

A dose–response metaanalysis examining circulating 25(OH)D level and mortality in prostate cancer patients was conducted for studies up to 2018 [111]. This metaanalysis, which included seven eligible cohort studies with 7808 cancer participants, supported a beneficial association. Specifically, the summary HR of prostate cancer–specific mortality for an increment of 20 nmol/L in circulating vitamin D level was 0.91, with 95% CI: 0.87–0.97, and the corresponding HR for all-cause mortality was 0.91 (95% CI: 0.84–0.98).

### 3.6 Lung cancer

The evidence for lung cancer survival is mixed. Most studies have measured postdiagnostic circulating 25(OH)D. A Norwegian study of 210 lung cancer cases [84] and a US study of 447 early-stage non–small-cell lung cancer cases [112] observed better survival with higher circulating 25(OH)D measured shortly after diagnosis. However, a small Chinese study of 87 cases of non–small-cell lung cancer did not observe a protective association [113]. Two studies have focused on advanced non–small-cell lung cancer with 294 cases [114] and 359 cases [115]; both did not report a difference in survival by circulating 25(OH)D levels. A recent study measured prediagnostic circulating 25(OH)D in 500 Finnish male smokers with lung cancer and did not find a significant association with survival. This study found suggestive protective effects for the histological subtypes adenocarcinoma and small cell carcinomas, but the estimates were based on a relatively small number of cases and were not statistically significant [116]. Another study found that having a higher prediagnostic blood level of 25(OH)D was associated with decreased lung cancer mortality in nonsmokers only [117].

### 3.7 Other cancers

A metaanalysis of studies conducted through December 2013 found that in addition to the protective association for breast and colorectal cancer described before, higher 25(OH)D levels were also associated with better lymphoma outcomes [80]. In addition, three studies have also found that higher 25(OH)D level collected at or near diagnosis was associated with better prognosis in melanoma patients [118–120]. A study of circulating 25(OH)D levels prior to chemotherapy initiation in advanced pancreatic cancer patients ( $n = 256$ ) observed no relationship between 25(OH)D levels and progression free or overall survival [121]. The advanced stage of disease (median survival was <6 months) and the fact that the majority of patients had deficient (44.5%) or insufficient (22.5%) levels of vitamin D (<20 and <30 ng/mL, respectively) may have limited the ability to observe an association. Finally, a metaanalysis of 17 cohort studies found a statistically significant inverse association between baseline 25(OH)D levels and cancer death; RR of total cancer mortality for the bottom compared with the top two-thirds of baseline 25(OH)D concentration was 1.25 (95% CI: 1.10–1.43) [122].

### 3.8 Studies using predicted 25(OH)D

Another approach to estimate circulating 25(OH)D levels when blood samples are unavailable has been to use a predicted surrogate of 25(OH)D. The predicted 25(OH)D approach may have some advantages and disadvantages compared with the use of a single measurement of circulating 25(OH)D in epidemiologic studies [123]. For example, it may be more logistically feasible to gather information on predictors of vitamin D (diet, sun exposure, physical activity, skin characteristics) on larger numbers of people, than to prospectively collect blood samples. However, there is substantial unexplained variability of 25(OH)D levels in these models, and they should not be used to measure absolute levels of 25(OH)D exposure. Instead, the predictive 25(OH)D levels are useful markers of relative rather than absolute vitamin D status. One analysis in the Health Professionals Follow-Up Study used a predicted surrogate of 25(OH)D to examine risk of total cancer mortality [124]. The analysis was based on a two-stage approach. In the first stage, circulating 25(OH)D was measured in a sample of 1095 men in this cohort. Then, geographical region, skin pigmentation, dietary and supplement intake, BMI, and physical activity (a surrogate of potential exposure to sunlight UV-B) were used to develop a predicted 25(OH)D score using multiple linear regression. In the second stage, the score was calculated for each of approximately 47,000 men, and then this

variable was examined in relation to subsequent risk of cancer mortality. A 25 nmol/L increment in predicted 25(OH)D was associated with a 29% reduction in total cancer mortality. The associations persisted even when adjusted for potential confounders such as smoking, and including body mass index and physical activity, which contribute to the predicted 25(OH)D. Associations were particularly strong for digestive cancers (colorectal, pancreatic, stomach, and esophageal cancers), oral/pharyngeal cancers, and leukemias.

Predicted 25(OH)D level at diagnosis was examined in relation to mortality among 1017 participants in the Nurses' Health Study and the Health Professionals Follow-Up Study who were diagnosed with colorectal cancer [125]. Those in the highest quintile compared with the lowest quintile of predicted 25(OH)D levels had a 50% decreased risk of cancer-specific mortality and a 38% decreased risk of overall mortality. These associations persisted even after adjusting for prediagnostic predicted 25(OH)D levels, suggesting that intervening on vitamin D status at cancer diagnosis could be relevant timing for improving prognosis.

### 3.9 Studies of vitamin D intake

Vitamin D intakes are relatively low in general, and in most populations, more vitamin D is made from sun exposure than is ingested from food sources. Nonetheless, vitamin D intake is an important contributor to 25(OH)D levels, especially in winter months in regions at high latitudes when it may be the predominant or sole contributor. Although a large proportion of cancer patients report using vitamin and mineral supplements, including those containing vitamin D, the literature assessing associations with cancer prognosis and mortality is still quite limited [50].

The After Breast Cancer Pooling project, a consortium of four cohorts of 12,019 breast cancer survivors from the United States and China, assessed postdiagnosis, post-treatment vitamin D supplement use, and breast cancer outcomes. No association was observed between vitamin D supplement use and overall breast cancer recurrence or mortality. However, when stratified by estrogen receptor (ER) status, vitamin D supplementation was associated with a significant 46% decreased risk of recurrence among ER positive, but not of ER negative tumors ( $p$ -interaction = 0.01) [126]. Dose and duration of vitamin D supplementation use was not assessed in this study. A study of women aged 55 years and older in the United Kingdom Clinical Practice Research Data-link found that women with breast, colorectal, lung, ovarian, or uterine cancer who had exposure to three or more prescriptions in the 5 years prior to cancer diagnosis for vitamin D supplements did not have better

survival compared with women who received one to two prescriptions [127]. Exposure misclassification of women's vitamin D status may be a major limitation to this study as there was no information on vitamin D supplements that were not prescription-based or other sources of vitamin D, actual adherence to the prescribed vitamin D, or vitamin D intake postdiagnosis. Additionally, the follow-up for mortality in this study was relatively short (median 30.4 months). Finally, a large US study of 2284 colorectal cancer patients (408 colorectal cancer-specific) observed no association between pre-diagnostic vitamin D intake and colorectal cancer-specific survival [128].

### 3.10 Randomized controlled trials

Randomized controlled interventions (RCTs) are considered the "gold standard" for establishing a causal association because ideally, through effective randomization, confounding can be largely eliminated as an alternative explanation for the observed results. Several trials have investigated various doses of supplemental vitamin D and overall cancer mortality as a secondary endpoint. Relatively few RCTs, for example, VITAL, had total cancer mortality as a prespecified secondary endpoint. Main limitations of the RCTs to date are that most have been conducted in older populations for non-cancer primary endpoints, and the sample sizes and study duration have been limited to assess cancer survival endpoints. The doses have varied, with some arguably too low. The mode of intervention included daily doses and less frequent high-dose bolus dosing. Some of the major RCTs are described briefly here:

- The Women's Health initiative (WHI) study was a randomized placebo-controlled trial of 400 IU vitamin D plus 1000 mg of calcium per day in 36,282 postmenopausal women. A post hoc analysis of the WHI study for cancer mortality showed that, although not statistically significant, women randomized to vitamin D and calcium had a reduced risk of cancer mortality (344 cancer deaths in the treatment group vs. 382 in the placebo group;  $RR = 0.89$ ; 95%  $CI = 0.77-1.03$ ) [129]. In a reanalysis of the WHI study limited-access dataset in 15,646 women (43%) who were not taking personal calcium or vitamin D supplements at randomization, the vitamin D and calcium intervention significantly decreased the risk of total, breast, and invasive breast cancers by 14%–20% and nonsignificantly reduced the risk of colorectal cancer by 17%, but no association was seen in women taking personal calcium or vitamin D supplements [130].
- In an RCT conducted in the United Kingdom, 2686 subjects aged 65–85 years old were randomized to



- receive either 100,000 IU of vitamin D<sub>3</sub> every 4 months (equivalent to 820 IU daily) or a placebo over a period of 5 years [131]. After treatment, the 25(OH)D level was 74.3 nmol/L in the vitamin D–treated group and 53.4 nmol/L in the placebo group (a ~21 nmol/L difference due to supplementation). There was a nonsignificant reduced risk of cancer mortality (63 cancer deaths in the treatment group vs. 72 in the placebo group; RR = 0.86; 95% CI = 0.61–1.20).
- Another UK study included 5282 subjects (85% women) aged 70 years and older who were randomized to daily 800 IU vitamin D<sub>3</sub>, 1000 mg calcium, both or placebo for 24–62 months with a 3-year follow-up period after the intervention. This study found a protective, but nonsignificant reduced risk of cancer mortality between those allocated vitamin D compared with those who did not receive vitamin D (HR = 0.85; 95% CI = 0.68–1.06) [132].
  - A large-scale randomized trial was conducted in the United States (Vitamin D and Omega-3 Trial) in which 20,000 participants have been randomized to take either 2000 IU of vitamin D<sub>3</sub> per day or a placebo over a 5-year period [133,134]. The primary endpoints were total cancer and major cardiovascular events; site-specific cancers and cancer mortality were secondary endpoints. While no effect was seen for cancer incidence, the hazard ratio was suggestive (HR = 0.83 (95% CI = 0.67–1.02) for cancer death [49]. The result was statistically significant when the first 2 years of follow-up (a prespecified secondary endpoint) was conducted. In addition, in a secondary analysis of this trial, the authors found a significant reduction in advanced cancers (metastatic or fatal) for those randomized to vitamin D supplementation compared with placebo (HR = 0.83; 95% CI = 0.69–0.99) [135].
  - Vitamin D and Longevity trial in the United Kingdom—is a feasibility trial of 1600 men and women randomized to 100,000 IU monthly (3300 IU/day) of oral vitamin D<sub>3</sub> or placebo—if successful, they will continue on to recruit 20,000 subjects (<http://vidal.lshtm.ac.uk/>).
  - The Finnish Vitamin D trial originally aimed to randomize 18,000 Finnish participants aged 60 (men) or 65 (women) and older to either 1600 IU/day or 3200 IU/day of vitamin D<sub>3</sub>, or placebo, but has since reduced the overall sample size to 2500 because of difficulties in recruitment and funding (CTG: NCT01463813). In this trial, vitamin D<sub>3</sub> supplementation did not lower the incidences of invasive cancer among older adults, possibly because most participants had sufficient vitamin D at baseline [136].
  - D-Health Trial [137] in Australia is a randomized placebo-controlled trial, with intervention for 5 years, and with 5 years of additional passive follow-up.

21,315 participants aged 65–84 were recruited and given monthly oral dose of 60,000 IU of cholecalciferol or placebo with the primary outcome being all-cause mortality and secondary outcomes being mortality from other causes including cancer. Vitamin D administration did not reduce cancer mortality in this trial (Hazard ratio = 1.15; 95% CI = 0.96–1.39) [138].

While overall the RCTs individually did not find significant results for vitamin D supplementation, a number of the studies found suggestive though not significant 10%–15% reductions in risk. Individually, the studies were not powered to show that such associations would be statistically significant. A recent meta-analysis considered the available RCTs of vitamin D supplementation and total cancer mortality for studies up to January 2022 [51]. Based on a total of six trials assessing total cancer mortality, the summary RR for vitamin D supplementation versus control group and total cancer mortality was 0.92 (95% CI, 0.82–1.03; *P* = .17). Interestingly, a significant inverse association emerged among studies that tested daily vitamin D supplementation (RR, 0.87; 95% CI, 0.78–0.96), but not among studies that tested infrequent large-bolus supplementation (RR, 1.05; 95% CI, 0.88–1.26). Of note, in the VITAL trial, a significant inverse association was observed only among normal weight individuals (RR, 0.58; 95% CI, 0.39–0.86), but not among overweight or obese individuals. No other study reported on the stratification by body mass index.

The results of these trials have the potential to provide more evidence for a causal association; however, they also are subject to the practical limitations discussed previously, including limited follow-up, late etiologic exposure period, the selection of an effective dose, and that they were designed for primary endpoints other than cancer mortality. There were also issues such as potential problems with compliance and use of vitamin D supplements other than those assigned in the study. All of these factors could obscure a true association, if one exists. Nonetheless, there is suggestive evidence from the RCTs of a late effect of vitamin D status on cancer mortality, which is supported by the observational data summarized in this chapter.

The role of vitamin D supplementation as an adjuvant to traditional cancer therapy is of interest. Zeichner et al. [139] conducted a retrospective observational study of 308 patients from a cancer center in Miami who received chemotherapy for nonmetastatic HER2+ breast cancer patients. They found that those patients who took vitamin D supplements had significantly improved disease-free survival compared with those on chemotherapy who did not take a vitamin D supplement.



The mean dose of vitamin D was 10,472 IU/week (1500 IU/day). These promising findings combined with the favorable risk–benefit ratio of vitamin D supplementation may warrant the design of RCTs among cancer patients. As vitamin D insufficiency and deficiency is prevalent among those diagnosed with cancer, RCTs could be designed in which high doses of vitamin D are provided to the randomized subjects to rapidly increase vitamin D stores at the time shortly before treatment to test the hypothesis that vitamin D status may favorably interact with treatment or promote better prognosis independent of treatment. Indeed, randomized trials of vitamin D supplementation as adjuvant therapy for melanoma [140,141] are in progress. However, it is important to note that the feasibility of these trials may be limited in some populations because many cancer patients are already taking or prescribed supplements [50,101].

#### 4. Genetic variation and gene expression in the vitamin D pathway

##### 4.1 Common variation

Several single-nucleotide polymorphisms (SNPs) associated with serum 25(OH)D levels have been identified, including variants in *GC* (or *DBP*), *CYP2R1*, and *DHCR7* [142] (see Chapter 60). If the association between higher 25(OH)D level and lower cancer mortality were causal, one would then expect to see a relationship between the SNPs that predict levels of 25(OH)D and cancer. Based on the concept of “Mendelian randomization,” a person’s genetic variation should not be confounded by other cancer risk factors—see Chapter 61. Limitations to these studies are that the SNPs identified only explain a small amount of variation (<5%) of 25(OH)D concentration, so most studies would not be powered to detect the corresponding small association with disease risk. Additionally, the relationship of the SNPs and 25(OH)D levels is complicated. For example, the SNPs most strongly associated with 25(OH)D levels were in the *DBP* (*GC* or *DBP*) and are also related to levels of this binding protein [143]. If these SNPs also affect the *DBP*’s affinity, genetically determined circulating 25(OH)D levels may not reflect true bioavailability. Thus, a null finding in these studies would not exclude a causal relationship between vitamin D and cancer.

In addition to Mendelian randomization studies, other studies have more generally assessed whether polymorphisms in genes involved in the vitamin D signaling pathway or its metabolism are associated with cancer prognosis or mortality. If relevant genetic polymorphisms are consistently associated with a

cancer, they lend additional support that vitamin D is the causal factor rather than confounding by another associated risk factor. In this section, we summarize studies that have assessed vitamin D–related genetic variation with mortality or survival in overall cancer, as well as for specific cancers, including colorectal, breast, prostate, lung, and melanoma. The subject of polymorphisms is further discussed in Chapters 60 and 61.

A study combining three Danish cohorts with almost 100,000 participants and 10,349 deaths (2839 from cancer) created an allele score from genetic variants in *DHCR7* and *CYP2R1* and found that each increase in allele score was associated with a 1.9 nmol/L lower 25(OH)D concentration [144]. As expected, the *DHCR7* and *CYP2R1* allele score was not associated with other lifestyle factors and thus is unlikely to be confounded. They did not examine SNPs in the *DBP* because of concern that the genetically determined 25(OH)D may not reflect bioavailable 25(OH)D. The odds ratio for a genetically determined 20 nmol/L lower plasma 25(OH)D concentration was 1.30 (95% CI = 1.05–1.61) for all-cause mortality and 1.43 (95% CI = 1.02–1.99) for total cancer mortality. The authors concluded that their results are compatible with the hypothesis that genetically low 25(OH)D concentrations may be causally associated with cancer-related mortality. However, other large studies did not find an association between genetically determined variation in 25(OH)D and cancer mortality [145].

Traditionally, Mendelian randomization studies assume a linear dose–response relation, that is, that the effect of an increase in 25(OH)D is constant across the range of 25(OH)D in the studied population. For example, a population increase for a level of 20–30 nmol/L has an equivalent benefit as an increase from 80 to 90 nmol/L. This assumption may not be realistic and would cause a loss of power if the association is nonlinear. One study examined nonlinear dose–response relationships of 25(OH)D concentrations with various mortality endpoints, including cancer mortality [146]. This study suggested that inverse associations between genetically predicted 25(OH)D concentrations and mortality outcomes were limited to the stratum of the population with low “nongenetic” related 25(OH)D concentrations (calculated as the residual from regression of 25(OH)D on the mean-centered genetic risk score). For total cancer mortality ( $n = 12,804$  events), there was no overall association of genetic 25(OH)D—a 10 nmol/L increment in genetic 25(OH)D had an odds ratio of 0.98 (95% CI = 0.93–1.02;  $P = .29$ ). However, the same OR for those with 25(OH)D levels <25 nmol/L was 0.81 (0.65–1.02;  $P = .09$ ), and for those with levels 25–49 nmol/L, it was 0.93 (0.87–1.00;  $P = .046$ ). This

finding suggests that circulating 25(OH)D is associated with decreasing risk of cancer mortality up to around 50 nmol/L with no further benefit thereafter, though the statistical methods are novel and need confirmation.

*Vitamin D receptor (VDR)* polymorphisms have traditionally been one of the most studied candidate genes related to the vitamin D pathway, though their functionality is not fully understood. *VDR* polymorphisms were not associated with colorectal cancer—specific or overall survival in two European studies; one study had 1202 colorectal cancer cases and 444 colorectal cancer—specific deaths [147], and the other combined two cancer cohorts for a total of 1397 cases and 336 CRC-specific deaths [148]. However, both of these studies only assessed a few of the most common *VDR* polymorphisms.

An analysis in a US cohort of men assessed 97 SNPs from seven vitamin D—related genes (*CYP27B1*, *CYP27A1*, *GC*, *VDR*, *RXR $\alpha$* , *CYP2R1*, *CYP24A1*) among 114 men with lethal prostate cancer and 1244 controls. The authors used a pathway-based approach to assess whether multimarker SNP-sets in the seven genes were associated with lethal prostate cancer and found that the seven gene SNP-set and the individual gene SNP-sets defined by the *VDR* and *CYP27A1* genes were associated with risk of lethal prostate cancer [106]. Measuring the joint effect of multiple SNPs could potentially capture more of the true association when compared with individual SNP analyses. However, a larger follow-up study conducted in the Breast and Prostate Cancer Cohort Consortium (BPC3) with 496 fatal cases of prostate cancer did not replicate these findings [149]. A smaller case-only study found suggestive associations for SNPs in *VDR*, *CYP27B1*, and *CYP24A1* with prostate cancer survival [150], but there was little overlap with the findings from the prior studies. These studies and one other [151] did not find evidence that SNPs specifically associated with serum 25(OH)D were significantly associated with fatal prostate cancer or prostate cancer prognosis.

A metaanalysis of 11 studies (seven with information on melanoma-specific survival) with 3137 melanoma patients from Europe and the United States found that the *GC* rs2282679 polymorphism, which was associated with lower 25(OH)D levels, was associated with increased melanoma-specific deaths but not overall survival [152]. One study investigating six *VDR* polymorphisms and melanoma outcomes did not find significant main effects with disease relapse or overall survival [118]. However, another study that assessed 38 *VDR* polymorphisms and melanoma survival, with 3566 cases and 254 melanoma-specific deaths, observed that eight SNPs had nominally significant associations, and one functional SNP that affects *VDR* protein expression (rs2239182) remained significant after correcting for

multiple testing [153]. A study of 305 melanoma patients assessed candidate SNPs in four vitamin D—related genes, including *VDR*, did not find an association with melanoma prognosis [154]. Recently, a comprehensive study in melanoma patients assessed 2669 SNPs in the vitamin D pathway and disease-specific survival. After replication, two SNPs, rs12512631 in *GC* and rs7850212 in *RXRA*, were significantly associated [155].

Candidate gene studies focused on *VDR* have found associations with breast cancer survival, but the results have not been consistent. Perna et al. assessed the association of four *VDR* variants with breast cancer survival in a sample of 498 patients and found that rs731236 (Taq1) was associated with prognosis; they did not find an association with the other three variants, including Fok1 [156]. A small study ( $n = 115$  cases) of the four common *VDR* variants found that the Fok1 FF genotype is linked to poor prognosis in African American women with breast cancer; no association was found for Taq1 or the other variants [157]. A more comprehensive study of 106 SNPs over eight vitamin D pathway genes, including *VDR*, found that five SNPs in *RXRA* and one SNP in *PLAUR* were associated with disease-free survival in 1029 patients with early-stage breast cancer; the association with several of the SNPs in *RXRA* with survival was modified by type of systemic treatment received [158].

*VDR* polymorphisms have been associated with better survival outcomes in both early-stage and advanced non—small-cell lung cancer (Fok1, Cdx2, Bsm1) [76,113,114,159]. A study based in China found that *VDR* Apa1 genotype was associated with different chemotherapy response in patients with advanced non—small-cell lung cancer [160].

More studies are needed to assess whether vitamin D—related genetic variants may modify the associations of vitamin D and cancer mortality. Only a few such studies exist, and the findings have not been consistent, making it difficult to draw conclusions. A study assessing vitamin D status, and *VDR* Fok1 or Bsm1, and colorectal cancer survival did not show evidence of interaction [147], whereas another study of colorectal cancer survival did find a significant interaction between the *VDR* SNP (rs11568820) and a *VDR* haplotype (GAGC), 25(OH)D levels, and CRC-specific mortality [81]. The BPC3 study mentioned previously did not find main effects of the SNPs but did observe suggestive interactions between SNPs in *GC* and *CYP2R1*, circulating 25(OH)D and the risk of fatal prostate cancer [149]. Of interest, several of the SNPs in these genes were associated with levels of circulating 25(OH)D. The risk of fatal prostate cancer was highest in those men who despite carrying alleles associated with higher 25(OH)D in the general population still had individually low circulating 25(OH)D levels. A

study of melanoma patients found that there was an increased risk of melanoma relapse in those with the *VDR* Bsm1 A allele in patients with low-circulating 25(OH)D levels only [118]. Finally, a study of 1514 participants found that a variant in *VDR* (rs7968585) modified the association of 25(OH)D and a composite clinical outcome (incident hip fracture, myocardial infarction, cancer incidence, and total mortality). This finding was further replicated through a metaanalysis of three independent cohorts [161].

Overall, the evidence for independent contributions of *VDR* genetic variants on cancer prognosis and mortality remains unclear. Initial findings could be false positives, particularly in light of multiple testing concerns and the inconsistent results between studies. Conversely, studies with smaller sample sizes could lead to false-negative findings, especially if they are underpowered to detect the more modest associations expected to be the case with more common genetic polymorphisms. Thus, it is crucial to replicate promising findings. Systematic metaanalyses or pooled data from cohort consortiums will be important to generate large enough sample sizes, especially to test specific cancer subtypes and the potential for interactions between genetic variants, vitamin D status, and cancers.

## 4.2 Gene expression

The bioavailability of 25(OH)D in target tissues could be mediated by the expression of vitamin D–related genes, and data on vitamin D–related gene expression and cancer mortality are emerging. Several vitamin D–related genes, including *CYP27B1*, *CYP24A1*, and *VDR*, have differential mRNA or protein expression in cancer, and this expression may be associated with tumor characteristics and prognosis, but more research is needed [162]. In general, higher *VDR* and *CYP27B1* and lower *CYP24A1* expression have been found to be associated with less aggressive or better cancer outcomes, but these findings are not conclusive [111,130,136]. Higher expression of the *VDR* in prostate tumor tissue has been associated with a 60% decreased risk of lethal cancer among men with prostate cancer [163]. Higher levels of *VDR* expression have been associated with more favorable tumor [164–167] and better survival in breast cancer [164,167,168] in some studies but not all [166,169]. A large study with over 1000 breast cancer patients and 271 deaths (130 breast cancer-specific deaths) over a median of 6 years of follow-up found that although *VDR* expression was inversely related to aggressive tumor characteristics, it was not associated with patient survival outcomes [166]. Another study of 718 women with invasive breast tumors found that positive *VDR*

expression was associated with both favorable tumor characteristics and lower risk of breast cancer mortality [168]. In lung cancer, higher *CYP27B1* expression was associated with better prognosis in non–small-cell lung cancer patients [170], high *CYP24A1* [171] was associated with poorer survival, and higher *VDR* [172] correlated with longer survival in lung adenocarcinoma. These changes in gene expression would be expected to correlate with better survival, if increased vitamin D signaling was beneficial, and decreased signaling was detrimental. In colorectal cancer, a study of 658 patients found that expression of *VDR* in tumor stromal fibroblasts was associated with better clinical outcomes (progression-free survival and overall survival) [173]. A study of 99 colorectal cancer patients found that higher *CYP24A* expression in tumor tissue was correlated with more aggressive tumors and worse overall and disease-free survival [174]. Brozyna et al. have conducted studies reporting that decreased *VDR*, *CYP27B1*, and *CYP24A1* expression were all related to markers of worse melanoma prognosis as well as survival [175–177].

## 5. Conclusions

Since 1980, the hypothesis that vitamin D influences cancer incidence or mortality has been of interest to many cancer researchers. Initial provocative studies were based mostly on ecologic data. In the recent three decades, a large number of studies have examined pre-diagnostic 25(OH)D in relation to cancer incidence and mortality, and some studies have examined 25(OH)D levels and prognosis in cancer patients. For cancer incidence, most studies based on 25(OH)D have not supported an association, except possibly for colorectal cancer. In addition, Mendelian randomization studies support that higher vitamin D status is associated with a reduced risk of ovarian cancer [178,179]. Other than for ovarian cancer, most Mendelian randomization studies have not supported an association of 25(OH)D with incidence of most cancers. RCTs, though limited to around 5 years of duration for the vitamin D intervention, have generally not supported a benefit of vitamin D supplementation on total cancer incidence, though evidence for different types of cancers has been limited.

Of more promise, the epidemiologic and supporting mechanistic and animal evidence indicate that vitamin D may have a role in reducing cancer mortality and cancer prognosis, especially in breast and colorectal cancer. The RCT data to date are not definitive, yet are consistent with an effect on cancer mortality. Overall, around a 15% reduction in total cancer mortality was observed in individuals, most without cancer at baseline, who were randomized to vitamin D and subsequently

followed for cancer mortality. The evidence is clearer for studies that use daily dosing of vitamin D rather than large dose bolus dosing. Intriguingly, a Mendelian randomization study found a statistically significant association between genetically low 25(OH)D levels and total cancer mortality, up to around 50 nmol/L, with no further benefit at higher levels [146]. Thus, an effect of vitamin D on cancer mortality and survival has received support and should be the focus of further study. However, the assumptions for non-linear Mendelian randomization may have been violated so findings for non-linearity require further confirmation [179A].

Although RCTs will provide the most definitive evidence, an ideal trial would be difficult to design as questions still remain about the dose response, the optimal level of 25(OH)D, what intakes of vitamin D would be required to achieve this level, and the most relevant timing of exposure. Some studies have not found a beneficial effect in later stage cancers, indicating that the relevant time period of exposure may be earlier on in disease development. But more studies are needed to determine if this is the case, and the relevant timing may differ by cancer or even cancer subtype. Beyond randomized trials, further large-scale observational studies would be useful. Studies are also needed to establish the role of modifying factors, such as vitamin D–related genetic variants, gene expression, or lifestyle factors.

Further, more research is warranted to examine if association of vitamin D and prognosis differs by cancer subtypes of disease [116,126]. It is important to note that molecular defects in the vitamin D pathway could affect response to vitamin D supplementation. For example, if a tumor loses its VDR or the ability to convert 25(OH)D to 1,25(OH)<sub>2</sub>D at some advanced stage, supplementation with vitamin D may not be efficacious at that point. Molecular defects may help us better understand why some cancers appear to be less sensitive to the actions of vitamin D and may suggest novel approaches. For example, in one study, high plasma 25(OH)D level was associated with lower risk of colorectal cancers that had an intense immune reaction but not in those without such a reaction [180]. A small study compared VDR expression and prognostic value in *BRCA1* mutated breast cancers versus sporadic breast cancer cases and found that VDR expression was detected in over 90% of triple negative *BRCA1* mutated breast cancer and was significantly (VDR:  $P < .001$ ) over-expressed in *BRCA1* mutated as compared with sporadic cancer cases. The data also suggested that VDR was more strongly associated with overall survival in *BRCA1* mutated cases [181].

In summary, according to the human studies to date, the influence of vitamin D on cancer progression and mortality may be a potentially fruitful avenue to pursue. It is still unclear whether the potential benefit for vitamin

D status on cancer mortality is in the prediagnostic stages by influencing tumor aggressive behavior, during treatment through positive interactions with therapies or in postdiagnostic stages by enhancing survival. Of course, these potential benefits are not mutually exclusive, and vitamin D could be acting at prediagnostic and postdiagnostic stages. The benefit of additional vitamin D is likely to be stronger in those with low levels (for example,  $<50$  nmol/L), but more study is needed. There is evidence of widely prevalent vitamin D deficiency in general and especially among those diagnosed with cancer; thus, the potential for public health impact of an intervention with vitamin D may be very large.

## 6. Summary points

- Vitamin D may reduce cancer incidence or mortality through various biologic mechanisms, which has stimulated many population-based studies of vitamin D status and cancer risk.
- Many epidemiologic studies have been conducted examining circulating 25(OH)D levels as well as other surrogates of vitamin D status and cancer risk.
- Higher circulating 25(OH)D levels have been associated with lower risk of colorectal cancer, but generally not other cancers.
- Higher circulating 25(OH)D levels have been associated with lower risk of cancer mortality and better prognosis in cancer patients. This is seen across a spectrum of cancer types.
- There have not been many RCTs that have specifically examined vitamin D supplementation for cancer risk or mortality. However, cancer incidence and mortality as a secondary endpoint has been reported in a number of RCTs of vitamin D.
- In metaanalyses of RCTs of vitamin D and cancer, vitamin D supplementation has generally not been associated with total cancer incidence, but it has been associated with about a 15% decrease in cancer mortality. This benefit is especially seen in trials that use daily dosing of vitamin D but not infrequent high-dose bolus protocols.
- Population-based genetic studies have been equivocal in support of a role of vitamin D and cancer.

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# Antiproliferative and immunoregulatory actions of vitamin D derivatives on hematological malignancies: control of differentiation, proliferation, and cell death

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## OBJECTIVES

- Present a general overview of differentiation-inducing effects of vitamin D derivatives (VDDs) on myeloid leukemia cells.
- Describe the signal transduction pathways and transcription factors involved in VDD-induced differentiation of myeloid leukemia cells.
- Discuss the regulatory effects of VDDs on cell cycle, proliferation, and survival of myeloid leukemia cells and the underlying mechanisms of these effects.
- Review the effects on lymphoid cells and the immunomodulatory activity of VDDs.
- Discuss the findings from model and clinical studies of VDDs and their combinations with other anticancer agents in hematopoietic malignancies.
- Emphasize significance of basic and clinical research of VDDs in hematopoietic malignancies: from bench to bedside.

## 1. Differentiation of myeloid leukemia cells by vitamin D derivatives

The studies of vitamin D derivatives (VDDs) as differentiation agents originated in 1981 when Tatsuo Suda's Laboratory showed that exposure of M1 mouse myeloid leukemia cells in culture to 1,25(OH)<sub>2</sub>D<sub>3</sub> induces these immature cells to differentiate into functional macrophages [1]. This important finding was followed by many studies confirming that acute myeloid leukemia (AML) blasts, murine or human cells [2,3], differentiate in response to treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>2</sub>, or their synthetic analogs (collectively referred to here as VDDs). Also, several other types of neoplastic cells show similar responses, including colon cancer, breast cancer, prostate cancer, neuroblastoma, osteosarcoma, squamous cell carcinoma, and malignant melanoma [4–6]. It was also found that tissue stem cells and normally developing immature progenitor cells can be induced to differentiate by 1,25(OH)<sub>2</sub>D<sub>3</sub>, including skeletal muscle satellite cells [7] and myoblasts [8], epidermal stem cells [9], keratinocytes [10,11], and hematopoietic cell precursors [12–14].



It is apparent that VDDs are powerful differentiating agents that target diverse cell types, but the physiological role of  $1,25(\text{OH})_2\text{D}_3$  in development remains to be fully determined. Data from vitamin D receptor (VDR) knockout (VDR-KO) mice suggest that  $1,25(\text{OH})_2\text{D}_3$  has little impact on normal myelopoiesis, though T lymphocytes exhibited abnormal responses in this model [12]. However,  $1,25(\text{OH})_2\text{D}_3$  increases adult hematopoietic stem and progenitor cell (HSPC) production and function in the zebrafish model *in vitro* and *in vivo* and enhances *in vitro* expansion and differentiation potential of  $\text{CD}34^+$  HSPCs from human umbilical cord blood [15] and bone marrow [16]. Human HSPCs and myeloid precursors were reported to have lower VDR expression than fully differentiated monocytes [16]. Interestingly, analysis of the gene expression data obtained by Metzeler et al. [17] demonstrated that VDR mRNA levels are also lower in leukemic cells from patients with undifferentiated or immature AML subtypes (AML-M0-M2) compared with AML subtypes with features of monocytic differentiation (AML-M4-M5) [16]. VDR-KO mice showed increased numbers of quiescent hematopoietic stem cells (HSCs) and leukemia stem cells, increased self-renewal, and myeloid differentiation block [16].

The interpretation of many *in vitro* studies with normal immature cells may be clouded by the use of high concentrations of  $1,25(\text{OH})_2\text{D}_3$  in significant excess over its physiological levels. However, it can be argued that concentrations of  $1,25(\text{OH})_2\text{D}_3$  in specific tissue niches in which precursor cells differentiate can be higher than the concentrations found in the plasma, because these niches may possess the  $1\alpha$ -hydroxylase (CYP27B1) enzyme, which permits the final hydroxylation of 25-hydroxyvitamin  $\text{D}_3$  ( $25(\text{OH})\text{D}_3$ ) to produce active hormonal  $1,25(\text{OH})_2\text{D}_3$  [18,19] (see Chapter 9). Currently, most studies utilize low nanomolar concentrations of  $1,25(\text{OH})_2\text{D}_3$  [19–21], and in this way, a clearer assessment of the physiological role of  $1,25(\text{OH})_2\text{D}_3$  in normal differentiation will perhaps be achieved.

## 1.1 Differentiation and its markers

Markers by which differentiated phenotypes of various cell types can be recognized are summarized, and sample references are provided in Table 86.1. HL60 human myeloblastic leukemia cells cultured with varying concentrations of  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-10}$  to  $10^{-7}$  M for 1–7 days) differentiate morphologically and functionally first toward monocytes then to macrophages. This is recognized by expression of CD14 (a coreceptor for bacterial lipopolysaccharide, cell surface marker of early monocytic differentiation) and the

cytoplasmic enzyme nonspecific esterase (NSE)/monocyte-specific esterase (MSE), then by expression of CD11b (alpha subunit of CR3 integrin, cell surface marker of monocyte/macrophage, and granulocyte differentiation). At this stage, AML cells with monocyte/macrophage phenotype become adherent to solid surfaces, develop pseudopodia, reduce nitroblue tetrazolium (NBT), and develop the ability to phagocytose particles [22,23]. It may be speculated that macrophage-like differentiation of AML cells may also reduce leukemia-induced bone loss, since it was shown that activated bone marrow  $\text{CD}11b^+$  monocytes/macrophages stimulate osteogenesis by promoting differentiation of mesenchymal stem cells into osteoblasts [24,25].

Abnormal immature white blood cells (leukemic blasts) from AML patients also respond to VDDs when cultured *in vitro*. They frequently undergo at least partial monocytic differentiation as assessed by CD14 and CD11b expression, NBT reduction, morphology, and phagocytic ability, and their clonal growth is often inhibited [26,27]. However, there can be differences between AML cells in continuous culture and AML blasts in primary culture. For instance, it was reported that several samples of leukemic blasts, including those that had a deletion of chromosome 7, differentiated toward monocytes when cultured with  $1,25(\text{OH})_2\text{D}_3$ , but the leukemic blast samples with mutations of FLT-3 (internal tandem duplication or missense mutation) did not differentiate under similar conditions [21]. Conversely, established AML cell lines with FLT-3 mutations can differentiate in response to VDDs [28,29], suggesting that culture conditions can alter the response to VDDs. Alternatively, the general mutational landscape, rather than one identified mutation, may determine the susceptibility of the cells to VDD-induced differentiation.

## 2. Signaling and execution of monocytic differentiation induced by VDDs

### 2.1 Activation of VDR as the initial VDD-induced signal for differentiation of AML cells

Cells that differentiate when exposed to  $1,25(\text{OH})_2\text{D}_3$  usually express VDR [117,118]. VDR belongs to the superfamily of nuclear receptors that are ligand-activated transcription factors. After ligation of  $1,25(\text{OH})_2\text{D}_3$ , VDR becomes stabilized [119], then forms a homodimer or a heterodimer with retinoid X receptor alpha ( $\text{RXR}\alpha$ ), and translocates from the cytosol to the cell nucleus [120]. Then, the dimeric complex binds to the vitamin D response elements (VDREs) located in the promoter regions of the target genes (see Chapters 10–12).

**TABLE 86.1** Examples of cellular models of differentiation induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDDs

Differentiation marker	Known features	Comments	References
<b>Hematopoietic cells (HL60, U937, THP-1, MOLM-13, M1, WEHI-3)</b>			
CD14	LPS cell surface-binding protein	Early monocytic differentiation	[30–34]
CD11b	Cell surface protein (integrin $\alpha$ M)	General myeloid differentiation	[35–38]
Nonspecific esterase	Cytoplasmic hydrolytic enzyme	Monocytic differentiation	[2,39–42]
Superoxide anion	Component of oxidative burst	General myeloid differentiation	[43–46]
Cathelicidin	Antimicrobial peptide	Innate immunity	[47]
Phagocytosis		General myeloid differentiation	[1,48–50]
Morphologic changes <sup>a</sup>			[2,41,42,50]
<b>Colon cancer cells (CaCo-2, SW480-ADH, HCT116), adenoma (LT97) and primary adenoma, and carcinoma cells</b>			
Alkaline phosphatase	Brush border-associated hydrolase	Intestinal and placental isozymes	[51,52]
Carcinoembryonic antigen (CEA)	Adhesion molecule	Early development protein	[53,54]
E-cadherin	Calcium-dependent cell adhesion molecule	Invasion suppressor	[55–57]
Calcium-sensing receptor (CaSR)	Calcium homeostasis	Putative tumor suppressor in colon	[6]
Morphologic changes <sup>a</sup>		Adhesive epithelial phenotype	[55,58]
<b>Osteoblast-like cells (MG-63, ROS 17/2.8, MC3T3-E1), primary dental pulp and dental follicle cells, iPSC-derived osteoprogenitors</b>			
Osteopontin	Bone sialoprotein I (BSP-1), adhesion molecule	Early osteoblastic differentiation	[59–61]
Osteocalcin	Osteoblast-specific noncollagenous protein	Late osteoblastic differentiation	[61–64]
Dentin sialophosphoprotein	Integrin-binding ligand N-linked glycoprotein	Odontoblastic differentiation	[65]
Dentin matrix protein-1	Tooth and bone acidic phosphoprotein	Odontoblastic differentiation	[64,65]
Alkaline phosphatase	Hydrolytic enzyme	Bone mineralization	[61,62,65,66]
Mineralization			[61,64,65]
<b>Prostate cancer cells (LNCaP, PC-3), primary prostate stem/progenitor cells</b>			
Prostate-specific antigen (PSA)	Serine protease	Secreted by prostate epithelial cells	[67–71]
Prostate-specific acid phosphatase protein tyrosine phosphatase	Prostate growth regulating enzyme		[68,72]
E-cadherin	Calcium-dependent cell adhesion molecule	Major epithelial cadherin	[69,73]
Type II transmembrane serine protease		Prostate epithelium protein	[71]
<b>Breast cancer cells (MCF-7, T47D, MDA-MB-231, MDA-MB-436, BT-20, SK-BR-3, UIISO-BCA-4)</b>			
E-cadherin	Calcium-dependent cell adhesion molecule	Major epithelial cadherin	[74,75]
Casein	Major milk protein		[76,77]
Lactoferrin	Iron-binding milk protein		[74]
Intracellular lipid droplets	Storage/precursor material		[76–79]
Morphologic changes <sup>a</sup>			[80]

Continued

**TABLE 86.1** Examples of cellular models of differentiation induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDDS—cont'd

Differentiation marker	Known features	Comments	References
	Reverts the myoepithelial features associated with more aggressive breast cancer		
<i>Neuroblastoma (LA-N-5), primary neural stem/progenitor cells</i>			
Acetylcholine esterase	Serine hydrolase	May regulate neurite outgrowth	[81–83]
Neuronal nuclei (NeuN)	Nuclear RNA-binding protein	Neuronal differentiation	[84]
Myelin basic protein	Lipid-interacting myelin protein	Oligodendrocyte differentiation	[85]
Galactosylceramidase	Lysosomal hydrolase	Oligodendrocyte differentiation	[84]
Neurite outgrowth			[82,83,86]
<i>Melanoma (B16), melanocyte precursor cells (NCC-melb4)</i>			
Tyrosinase	Copper-containing oxidase	Key enzyme in melanin synthesis	[87–89]
<i>Squamous cell carcinoma (SCC13, SCC25, SCC 2/88, SCCVII-SF)</i>			
Keratin 1	Fibrous scleroprotein	Structural skin component	[90]
Transglutaminase	Calcium-dependent cross-linking enzyme	Keratinocyte-specific form	[91]
Involucrin	Glutamine-rich transglutaminase substrate	Cornified cell envelope constituent	[90,92–94]
E-cadherin	Calcium-dependent cell adhesion molecule	Major epithelial cadherin	[95]
<i>Keratinocytes</i>			
Transglutaminase	Calcium-dependent cross-linking enzyme	Keratinocyte-specific form	[91,96–99]
Keratin 1	Fibrous scleroprotein	Early differentiation marker	[98,99]
Keratin 10	Fibrous scleroprotein coexpressed with keratin 1	Early differentiation marker	[98,99]
Involucrin	Glutamine-rich transglutaminase substrate	Cornified cell envelope constituent	[92,97–102]
Cystatin A	Cysteine proteinase inhibitor	Cornified cell envelope constituent	[103,104]
Loricrin	Late differentiation marker		[98,99]
Filaggrin	Late differentiation marker		[98,99]
Cornified envelope formation			[96,105]
E-cadherin	Calcium-dependent cell adhesion molecule	Major epithelial cadherin	[106]
Morphologic changes <sup>a</sup>			[11]
<i>Myoblasts (C2C12), embryonic myocardium cells (H9c2)</i>			
MyoD	Myogenic transcription factor	Early myogenic differentiation	[107]
Desmin	Subunit of intermediate filaments	Intermediate differentiation	[107]
Myosin	Contractile protein	Late differentiation	[108,109]
Creatine kinase	ATP metabolizing enzyme		[109,110]
Cardiac troponin	Ca <sup>2+</sup> -binding contraction regulatory complex	Cardiomyocyte differentiation	[111]
Morphologic changes <sup>a</sup>		Formation of myotubes	[107,111]

**TABLE 86.1** Examples of cellular models of differentiation induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDDs—cont'd

Differentiation marker	Known features	Comments	References
<i>Vascular smooth muscle cells transdifferentiating into osteoblasts (primary cultures)</i>			
Osteopontin	Bone sialoprotein I (BSP-1), adhesion molecule	Osteoblastic differentiation	[112]
Alkaline phosphatase	Hydrolytic enzyme	Osteoblastic differentiation	[112–115]
Bone morphogenetic protein 2	Differentiation-inducing cytokine	Osteoblastic differentiation	[115]
Msh homeobox 2	Osteoblast-specific transcription factor	Osteoblastic differentiation	[115]
Osterix	Osteoblast-specific transcription factor	Osteoblastic differentiation	[114]
Receptor activator of NF-κB ligand	Osteoblast surface protein	Osteoblastic differentiation	[114]
Runt-related transcription factor 2	Osteoblast-specific transcription factor	Osteoblastic differentiation	[114–116]
Mineralization		Bone nodule formation	[114,116]

<sup>a</sup>Morphologic changes can be recognized in many forms of differentiation.

Recent findings obtained using next-generation sequencing (NGS) approaches, e.g., chromatin immunoprecipitation sequencing (ChIP-seq), in various cell types, including THP-1 human monocytic leukemia cells have expanded this classical scheme of VDR action to more complex epigenome-, genome-, and transcriptome-wide models [121–124]. In particular, it has been demonstrated that unliganded VDR already binds to a limited number of persistent loci within accessible chromatin and may act as first contact points of 1,25(OH)<sub>2</sub>D<sub>3</sub> with the genome [124]. In THP-1 cells, stimulation with 1,25(OH)<sub>2</sub>D<sub>3</sub> leads to a substantial (up to 10-fold) increase in genome-wide VDR binding via support of pioneer factors, such as PU.1, CEBPα, or GABPα (reviewed in Ref. [125]). Moreover, PU.1 showed a far more frequent colocation with VDR than RXR, and only the minority of the VDR-binding sites contained classical VDREs, which are formed by direct repeats spaced by three nucleotides (DR3) and bind VDR-RXR heterodimers [126]. Using RNA-seq, it has been shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> induces a rapid (2.5–6 h) upregulation of ~370 genes in HL60 cells [127] and ~520 genes in THP-1 cells [128]. Further bioinformatics analysis predicted that in THP-1 cells, ~75% of the early-responsive genes and ~60% of those responding at a longer time point (24 h) are primary VDR targets [124,128]. The relation of thousands of genomic VDR-binding sites to several hundred primary VDR target genes remains largely unclear (see Chapters 10–12).

VDR expression has been detected in various types of mammalian cells [118], such as normal and leukemic hematopoietic cells, or stromal cells from the bone marrow [129–131]. The cellular amount of VDR transcript and

protein varies among the tissues and cultured cell lines, ranging from a few copies per cell, up to several dozen copies per cell [132]. VDR is expressed in both myeloid and lymphoid cell lineages and can be detected in various types of white blood cells, such as monocytes, macrophages, neutrophils, lymphocytes, dendritic cells (DCs), and platelets [129,130,133,134]. Interestingly, circulating monocytes have higher levels of VDR than tissue macrophages [135], which represent a more advanced stage of VDD-induced differentiation. Mature DCs showed lower levels of VDR than immature DCs or monocytes [130]. The requirement of a functionally active VDR for the differentiating action of 1,25(OH)<sub>2</sub>D<sub>3</sub> was confirmed by the studies on normal blood progenitor cells from VDR-KO mice that failed to differentiate into monocytes/macrophages in response to 1,25(OH)<sub>2</sub>D<sub>3</sub> [12]. Moreover, studies on VDR-KO mice with truncated or unliganded VDR showed that myeloid DCs generated ex vivo from bone marrow progenitors develop and function normally [136].

A large number of myeloid leukemia cell lines, blocked at various stages of maturation, express mRNA for VDR, although at different levels [129,137]. The presence of VDR is strictly required for VDD-induced monocyte/macrophage differentiation of AML cells [138,139], as well as normal mononuclear spleen cells and myeloid stem cells [12,140]. For instance, transfection of VDR conferred differentiation responsiveness to 1,25(OH)<sub>2</sub>D<sub>3</sub> in WEHI-3B D<sup>+</sup> murine myelomonocytic cells, which lack inducible VDR expression [138]. In VDR knockout mice, the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), but not 1,25(OH)<sub>2</sub>D<sub>3</sub> [12] or 19-nor-1,25-dihydroxyvitamin D<sub>2</sub>



(paricalcitol) [140], induced differentiation of bone marrow-committed myeloid stem cells to monocytes/macrophages, which indicates the requirement of VDR for 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced monocyte/macrophage differentiation. These findings are supported by the fact that CRISPR/Cas9-mediated VDR-KO in THP-1 cells led to a complete loss of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced gene regulation, indicating that transcriptome-wide effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> were entirely mediated by VDR [128]. The sensitivity of AML cells to VDD-induced differentiation depends on VDR protein levels. Cells with low initial level of VDR are resistant, but this may be overcome when VDR is upregulated by other compounds, such as *all-trans* retinoic acid (ATRA) [137]. Moreover, initial level of VDR protein can be elevated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [120]. In addition, MEG-01 human megakaryoblastic leukemia cells matured to megakaryocytes by TPA showed increasing VDR expression [141]. Inhibitors of MEK1/2 (PD98059) and PI3K (LY294002) reduced 1,25(OH)<sub>2</sub>D<sub>3</sub>-stimulated VDR upregulation and nuclear translocation, suggesting the involvement of MAP and PI3K kinases in these events [120]. The essential role of the p38 and JNK stress pathways and the downstream transcription factor activator protein-1 (AP-1) in upregulation of VDR expression was demonstrated in breast cancer cells [142]. Activation of the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2) has also been shown to result in VDR upregulation in AML cells [143,144].

The classical paradigm of 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling is that the gene expression-modulating actions of VDR-RXR heterodimers transmit the differentiation signals to basal transcription machinery by interacting with VDRE sequences of the genome [145], and details are described in greater detail in Chapters 10–12. In an outline, a large assortment of nuclear receptor coactivators, such as DRIP/mediator and SRC/p160 and corepressors (e.g., SMRT and N-CoR) has been identified, which provides positive or negative regulation of the VDR transcriptional activity [146,147]. In addition to the “classic” VDRE sequences that transactivate vitamin D-responsive gene transcription, the “negative” VDREs have also been characterized that inhibit transcription of certain genes, e.g., parathyroid hormone gene [148]. Interestingly, in myeloid leukemia cells, promyelocytic leukemia zinc finger (PLZF), and the chromosomal translocation products, promyelocytic leukemia-retinoic acid receptor alpha (PML/RAR $\alpha$ ) and PLZF/RAR $\alpha$  also repress the differentiating action of VDR by binding and sequestering the VDR [149,150].

How the initial VDD-induced gene expression leads to the acquisition of a new functional phenotype, i.e., differentiation, is one of current mysteries, as among the multiple known direct target genes of VDR [128] only a few have been linked to the differentiating actions of

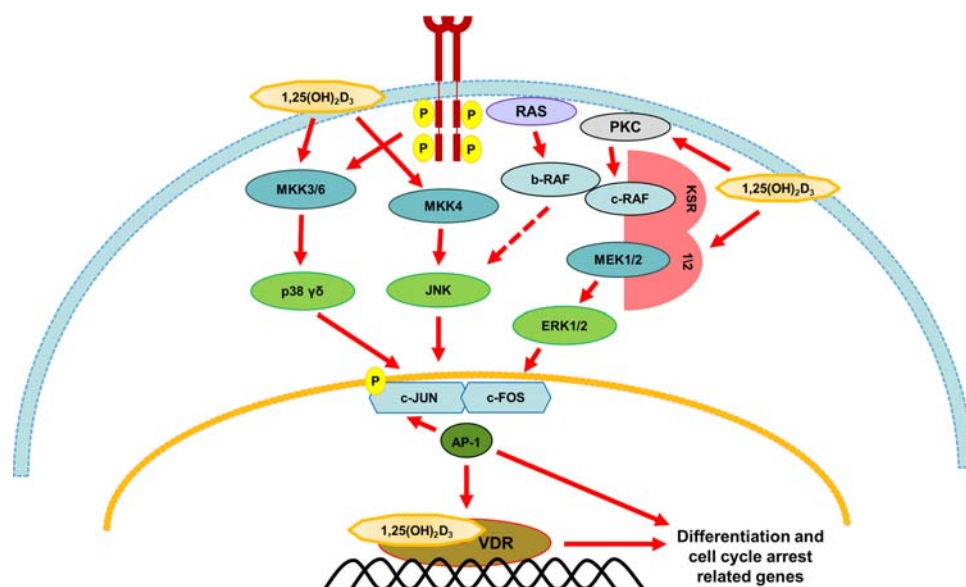
1,25(OH)<sub>2</sub>D<sub>3</sub> in tissues other than bone. In myeloid cells, these include CD14 [32,151], heterodimeric cell surface receptors, comprised of one  $\alpha$  subunit (CD11a, CD11b, or CD11c) and a common  $\beta$ -chain (CD18) [151], p21<sup>Cip1</sup> [151–153], as well as KSR1 and KSR2 [154,155]. Recent studies have demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> regulates the expression of multiple transcription factors in AML cells [127,128]. For instance, among the genes differentially expressed by 1,25(OH)<sub>2</sub>D<sub>3</sub> in THP-1 cells and predicted to be primary VDR targets, 47 were found to code for transcription factors [128]. In this context, the monocyte-like differentiation of HL60 cells treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> may require AP-1 complexes, which bind to the TPA-responsive element (TRE) of the promoter region in human VDR [156]. However, with regard to differentiation of most cell types, the links from VDR-initiated events to the downstream targets of 1,25(OH)<sub>2</sub>D<sub>3</sub> still need to be found.

## 2.2 Differentiation signaling pathways activated by VDDs in AML cells

Exposure of hematopoietic cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> controls hundreds of genes, including those responsible for the regulation of cellular proliferation, differentiation, apoptosis, and angiogenesis [157], also reviewed in Ref. [158]. Using a genome-wide analysis of VDR-binding sites in THP-1 human monocytic leukemia cells, 2340 such sites were identified. Of those, 1171 and 520 occurred uniquely with and without 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment, respectively, while 649 were common. Ligand treatment revealed 638 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes associated with immunity and signaling [159]. The genes CD14 and thrombomodulin (THBD) are upregulated primary vitamin D targets and were found to be suitable markers for vitamin D signaling in peripheral blood mononuclear cells and adipose tissue of individuals supplemented with vitamin D<sub>3</sub> [32]. The authors suggested that these biomarkers allow the classification of patients into those who may benefit from vitamin D<sub>3</sub> supplementation and others who may not. Modulation of these genes by 1,25(OH)<sub>2</sub>D<sub>3</sub> may not always be a direct effect on transcription of target genes but can reflect the entire process of differentiation associated with a series of interacting transcription factors. This and some highlights of the following discussion are illustrated in Fig. 86.1 and summarized in Table 86.2.

## 2.3 Protein kinase C signaling

A number of early studies linked several isoforms of protein kinase C (PKC) to differentiation [160]. Following the observation by Martell, Simpson, and Taylor [161] that treatment of HL60 cells by



**FIGURE 86.1** The MAPKs pathways that are upregulated by  $1,25(\text{OH})_2\text{D}_3$  in AML cells. The ERK and JNK pathways have positive effects on differentiation [183,251], while the p38 MAPK pathway may have a dual effect on differentiation. Several lines of evidence demonstrate that p38 $\alpha$  and p38 $\beta$  have an inhibitory effect on monocytic, but not granulocytic differentiation of HL60 cells [606,654], while p38 $\gamma$  and p38 $\delta$  may positively modulate monocytic differentiation of these cells [655].  $1,25(\text{OH})_2\text{D}_3$  can amplify the RAF-MEK-ERK pathway by direct transcriptional upregulation of kinase suppressor of RAS-1 (KSR1) [154], which acts as a scaffold that coordinates signaling along the RAS/ERK signaling module [656] or as an active kinase that phosphorylates c-RAF [657]. KSR2, a close homolog of KSR1 that is also directly upregulated by  $1,25(\text{OH})_2\text{D}_3$  [155], may have a similar role in the activation of the RAF-MEK-ERK pathway [658]. Also shown is the potential role of the AP-1 transcription factor, which acts as an intermediary positive effector of  $1,25(\text{OH})_2\text{D}_3$  signals by upregulating the expression of VDR [37]. Although only c-JUN and c-FOS are illustrated here, AP-1 can have other, interchangeable components. B-RAF has been shown to activate JNK, perhaps as a homodimer or a heterodimer with c-RAF (aka RAF-1) [464].

$1,25(\text{OH})_2\text{D}_3$  increases cellular TPA receptors, which implies increased PKC abundance, Hannun's laboratory showed that  $1,25(\text{OH})_2\text{D}_3$  increases the mRNA for isoforms  $\alpha$  and  $\beta$  of PKC in these cells [162]. These results were duplicated in many other laboratories, and PKC inhibitors or antisense oligonucleotides to this enzyme were demonstrated to interfere with  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation [163], further implying a role for at least some isoforms of PKC in differentiation induced by  $1,25(\text{OH})_2\text{D}_3$ . If its role could be established, PKC would provide an important regulatory element in a logically pleasing sequence of events that lead from an exposure of a cell to  $1,25(\text{OH})_2\text{D}_3$  toward differentiation. First, the lipid-soluble  $1,25(\text{OH})_2\text{D}_3$  may interact with cell membrane lipids or activate membrane-associated phospholipases [164] directly or through the still elusive membrane receptor, to generate a phospholipid second messenger, such as inositol 1,4,5-triphosphate ( $\text{IP}_3$ ) and 1,2-diacylglycerol (DAG).  $\text{IP}_3$  then releases calcium from the endoplasmic reticulum and thus promotes an increase in intracellular calcium ( $[\text{Ca}^{2+}]_i$ ).  $\text{Ca}^{2+}$  influx mediated by store-operated channels was also found to contribute to  $[\text{Ca}^{2+}]_i$  elevation in  $1,25(\text{OH})_2\text{D}_3$ -treated HL60 cells [165]. As the result of raised  $[\text{Ca}^{2+}]_i$  and DAG concentrations, several PKC isoforms can be activated [166,167], and the signal can be propagated further by

activating regulators of other signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathway. Known examples of such links are the phosphorylation of c-RAF by PKC $\alpha$  in chick myoblasts [168] and the translocation of MAPK ERK1/2 to the nucleus in HL60 cells [169]. Differentiation of HL60 cells in response to  $1,25(\text{OH})_2\text{D}_3$  is accompanied by increased levels of PKC- $\beta$ , and this differentiation can be inhibited by a specific PKC inhibitor, chelerythrine chloride [170]. Other potential roles of PKC in  $1,25(\text{OH})_2\text{D}_3$  signaling are the regulation of VDR [171] and a major VDD-metabolizing enzyme, 24-hydroxylase (CYP24A1) [172,173] by PKC activation.

However, several major difficulties have so far precluded a full assessment of the role of PKC activity in differentiation. These include its presence in multiple isoforms with overlapping properties, and the fact that full inhibition of cellular PKC activity is usually incompatible with cell survival.

## 2.4 PI3-K/AKT/mTOR pathway

Participation of the phosphoinositide 3-kinase (PI3-K) pathway in VDD-induced monocytic differentiation was first noted by Hmama et al. by the finding that antisense

**TABLE 86.2** Molecular targets of VDDs in leukemic cells.**Cell cycle/Apoptosis<sup>a</sup>**

CYC A1 ↓

CYC D1 ↓

CYC E ↓

CDKN1A (p21<sup>Waf1</sup>) ↑CDKN1B (p27<sup>Kip1</sup>) ↑

CDKN2A (p16-INK4A) ↑

CDKN2B (p15-INK4B) ↑

CDKN2C (p18-INK4C) ↑

BCL-2 ↓

BIM ↓

**Differentiation markers**

CD11b ↑

CD14 ↑

NSE activity ↑

NBT reduction ↑

**Oncogenes**

c-MYC ↓

DEK ↓

FLI1 ↓

c-FMS (M-CSFR) ↑

**Tumor suppressors**

PTEN ↑

BTG ↑

Rb ↑

**Kinases**

PKC levels ↑

PI3-K activity ↑

AKT activity ↑

ERK 1/2 activity ↑

JNK 1/2 activity ↑

p38  $\alpha\beta\gamma\delta$  activity ↑

KSR-1,-2 activity ↑

ERK-5 activity ↑

COT-1 activity ↑

**Transcription factors**

VDR ↑

C/EBP $\beta$  ↑

PU.1 ↑

IRF8  $\beta$  ↑

**TABLE 86.2** Molecular targets of VDDs in leukemic cells.—cont'd

HoxA10 ↑
HoxB4 ↑
AP-1 ↑ <sup>b</sup>
PPARδ ↑
JUN D-binding activity ↑
DRIP ↑
ETV7 ↓
<b>Feedback control</b>
CYP24A1 ↑
<b>Immunity</b>
Cathelicidin ↑
β-Defensin ↑

<sup>a</sup>Regulation of expression or activity may occur either directly or as a consequence of differentiation. See text for details.

<sup>b</sup>Putative components of AP-1 complex are c-JUN, ATF-2, JUN B, and FOS B.

oligonucleotides against PI3-K abrogate CD14 expression in THP-1 and HL60 cells, as well as in normal monocytes [174]. More recent reports include the observation that in HL60 cells, the PI3K inhibitor LY294002 blocks 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced increases in NBT reduction, MSE activity [175], and differentiation [176]. Furthermore, the PI3-K/AKT pathway appears to promote cell cycle progression in HL60 cells within 48 h of exposure to 1,25(OH)<sub>2</sub>D<sub>3</sub> [177]. Also, in HL60 cells, PI3-K inhibitors can synergize with 1,25(OH)<sub>2</sub>D<sub>3</sub> to induce cell cycle arrest, and this is associated with a synergistic upregulation of the p27<sup>Kip1</sup> cyclin-dependent kinase inhibitor, as illustrated in Fig. 86.2 [177]. It is possible that this effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell proliferation contributes to the survival enhancing effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and other VDDs in AML cells [178]. Interestingly, 1,25(OH)<sub>2</sub>D<sub>3</sub> and the AKT inhibitor MK-2206 were found to synergistically induce apoptosis in steroid-resistant T-ALL cells [179].

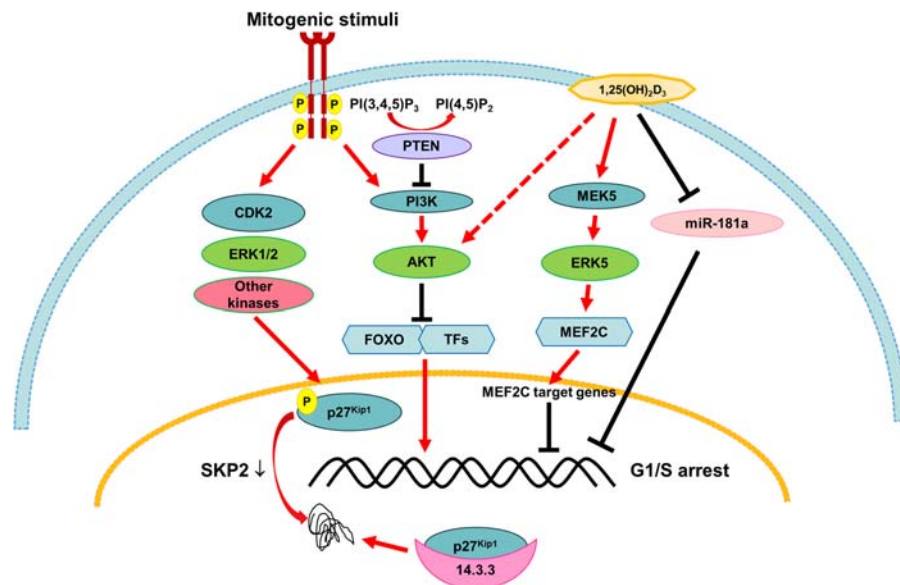
In addition, treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> or its synthetic analog 21-(3-methyl-3-hydroxy-butyl)-19-nor D<sub>3</sub> (Gemini-19-nor) for 4 days induced the expression of PTEN, which could block the PI3-K/AKT pathway, resulting in differentiation, cell death, or inhibition of growth of HL60 cells [180]. The role of this pathway in VDD-induced differentiation was further explored by the use of everolimus (aka RAD001), a rapamycin analog that inhibits AKT/mTOR complex-1 (mTORC1) signaling. It was found that everolimus potentiates the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the differentiation and proliferation of U937 cells [181]. More recently, it has been reported that the rapamycin-insensitive mTOR complex-2

(mTORC2) can function as an upstream regulator of the PI3-K/ATK pathways in HL60 cells induced to differentiate by 1,25(OH)<sub>2</sub>D<sub>3</sub> and that silencing of the rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) by siRNA suppressed the ability of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated cells to reduce NBT [175]. Interestingly, 1,25(OH)<sub>2</sub>D<sub>3</sub> was shown to induce both the expression of CD14 and activation of the mTOR signaling pathway in U937 and THP-1 cells differentiated into macrophages by the PKC activator TPA [182]. This was prevented by mTOR inhibitors PP242 and Torin1 or by siRNA-induced silencing of either regulatory-associated protein of mTORC1 (Raptor) or RICTOR, indicating that both mTORC1 and mTORC2 are important for 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced expression of CD14 in myeloid leukemia cells. The potential of VDD-regulated intracellular signaling, including the PI3-K/AKT pathway as targets for myeloid leukemia therapy, has been reviewed [158].

## 2.5 MAPK pathway

This pathway may be crucial for VDD-induced differentiation of AML cells. Exposure of either HL60 or NB4 cells to differentiation-inducing concentrations of VDDs causes activation and nuclear translocation of the ERK, JNK, and p38 MAPK proteins [37,169]. In these experiments, 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulated the transient (10 min–48 h) phosphorylation of ERK1/2 in HL60 cells. After 24 h, the level of phosphorylated ERK decreased to basal levels, while differentiation





**FIGURE 86.2** Regulators of p27<sup>Kip1</sup> expression during G1/S progression at both translational and posttranslational levels. The main form of regulation of the p27<sup>Kip1</sup> protein levels in most mammalian cells is proteasome-dependent degradation, as illustrated in the lower part of this figure. In proliferating cells, p27<sup>Kip1</sup> is phosphorylated by several kinases (originally proposed to be CDK2 and ERKs [378]), then ubiquitinated by SKP2 [386], which leads to its degradation. In addition, p27<sup>Kip1</sup> can be destabilized by phosphorylation on non-CDK sites [390–392] or by cytoplasmic localization through binding to proteins such as 14-3-3 [659,660]. However, there is an important contribution of transcriptional control. For instance, in myeloid cells, translation of p27<sup>Kip1</sup> mRNA can be inhibited by miR-181a, resulting in G1/S block [20,393]. Conversely, upregulation of p27<sup>Kip1</sup> expression can be transcriptionally activated by the forkhead transcription factors (TFs), such as AFX (FOXO4) [381,382], as well as the AP-1 transcription factor (not shown here) [384]. The FOXO activity is under the control of the PI3-K/AKT pathway, which in turn is regulated by the tumor suppressor PTEN, important in human malignancies. In this figure, the double helix shape in the lower part represents the human genome, as the precise transcription start site of p27<sup>Kip1</sup> has not been clearly identified. Various start sites for this gene have been described, and the major site was found to produce a GC-rich 5'-UTR of 472 nucleotides [385,661,662]. The MEK5/ERK5 pathway and the direct ERK5 downstream target transcription factor MEF2C [190] are upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [33]. Inhibition of MEK5/ERK5 [192] or the upstream activator COT1/TLP2 kinase [191] leads to upregulation of p27<sup>Kip1</sup> and proliferation/cell cycle arrest in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated AML cells.

continued over an additional 48 h [183]. In another report, phosphorylated c-RAF, MEK1/2, and ERK1/2 were detected in HL60 cells at least 72 h after treatment with  $5 \times 10^{-9}$  M 1,25(OH)<sub>2</sub>D<sub>3</sub> [184]. Further, PD98059, an ERK1/2 inhibitor, blocked the 1,25(OH)<sub>2</sub>D<sub>3</sub>-stimulated differentiation of HL60 cells [184]. In addition to inducing long-term MAPK activation, VDDs are also capable of rapid stimulation of this and other signal transduction pathways. This is thought to be mediated by caveolae-associated nuclear VDR, probably in conjunction with nonnuclear 1,25(OH)<sub>2</sub>D<sub>3</sub> receptors, such as membrane-associated rapid response steroid (MARRS) or protein disulfide isomerase family A member 3 (PDIA3), and caveolin-1 (reviewed in Refs. [185–188]. For instance, 1,25(OH)<sub>2</sub>D<sub>3</sub> was found to markedly increase phospho-ERK1/2 levels in HL60, NB4, U937, and THP-1 cells following 60 min of incubation [189]. This effect was blocked by pharmacological antagonists of nuclear VDR and did not appear to require heterodimerization with RXR $\alpha$ . Inhibitors of the SRC tyrosine kinase, RAS, RAF, and PKC $\alpha$  also abrogated the 1,25(OH)<sub>2</sub>D<sub>3</sub>-stimulated increase in ERK kinase activity. These results support the notion that the

plasma membrane-associated nuclear VDR can mediate rapid nongenomic 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling in AML cells, SRC, and PKC being upstream regulators of the MAPK pathway [189]. Other studies have also demonstrated that PKC is an upstream regulator of the RAF/MEK/ERK pathway [168,169].

A less well-studied MAPK is ERK5. This kinase overlaps the functions of ERK1/2 in cell proliferation and survival, functioning in a manner distinct from ERK1/2 in human AML cells induced to differentiate by 1,25(OH)<sub>2</sub>D<sub>3</sub>. Using inhibitors of ERK1/2 and of MEK5/ERK5 at concentrations specific for each kinase in HL60 and U937 cells, it was found that ERK5 and its direct downstream target transcription factor MEF2C [190] are upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> in parallel with monocytic differentiation [33]. Earlier experiments in these cell lines demonstrated that ERK5 phosphorylation (activation) by an exposure to 1,25(OH)<sub>2</sub>D<sub>3</sub> and, particularly, by its combination with the plant antioxidant silibinin, was, at least partially, dependent on the activity of an oncogenic COT1/TLP2 kinase [191]. Notably, inhibition of either COT1/TLP2 [191] or MEK5/ERK5 [192] resulted in upregulation of p27<sup>Kip1</sup>

and proliferation/cell cycle arrest in  $1,25(\text{OH})_2\text{D}_3$ -treated AML cells. This  $1,25(\text{OH})_2\text{D}_3$ -regulated pathway is illustrated in Fig. 86.2. Interestingly, the transcription factor C/EBP $\beta$  was found to be positively regulated, while C/EBP $\alpha$  was negatively regulated by ERK5 upon cell exposure to  $1,25(\text{OH})_2\text{D}_3$  [190,192].

Upstream from ERKs in the MAPK pathway are kinase suppressors of RAS-1 and RAS-2 (KSR1-2), which phosphorylate c-RAF and act as scaffolds, and increase the efficiency of signaling by c-RAF [154,155]. These two genes have an upstream promoter containing a functional VDRE motif. Knockdown of KSR-2 blocked  $1,25(\text{OH})_2\text{D}_3$ -induced myeloid differentiation. Signaling by RAF-1 is required for the later stage of  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation and requires p90 RSK, which is either directly or indirectly phosphorylated by c-RAF [193].

## 2.6 ERK5 and VDD-induced progression of monocytic to macrophage differentiation

As described before, a prolonged exposure of cultured AML cells to VDDs results first in the emergence of monocytic phenotype (CD14 and NSE positivity), then macrophage-like features (increased CD11b expression, characteristic morphological changes). This can be accelerated by the addition of a retinoid to the VDD, as evidenced by the appearance of M2 macrophage marker genes, such as CD163, ARG1, and IL-10, increased surface CD163 expression, and IL-10 protein secretion [194]. Mechanistically, other data support the hypothesis that ERK5 negatively regulates the expression of M-CSFR, and thus has a restraining function on macrophage differentiation, resulting in a prolonged phase of monocytic phenotype [50]. This may be one example of the complexity of differentiation programs, which are currently being apprehended more fully.

## 2.7 WNT/ $\beta$ -catenin signaling

The WNT/ $\beta$ -catenin signaling pathway has tied to both  $1,25(\text{OH})_2\text{D}_3$  signaling and leukemia development. This pathway is well established in the regulation of development and differentiation, and its dysregulation has been demonstrated in a number of neoplastic diseases including leukemias and lymphomas [195–198]. WNT ligands bind to a receptor complex containing one of the Frizzled proteins (FZD) and either LDL receptor-related protein 5 or 6 (LRP5/6), promoting the eventual translocation of the transcriptional activator  $\beta$ -catenin into the nucleus [197,198]. The activation of the noncanonical ( $\beta$ -catenin-independent) WNT pathway involves additional coreceptors, such as the receptor Tyr kinase-like orphan receptor family (ROR1

and ROR2), the receptor tyrosine kinase RYK, and the protein Tyr kinase 7 (PTK7). The noncanonical signaling is mediated by small GTPases RhoA, Daam1, and ROCK, acting on cytoskeleton remodeling, or by phospholipase C/PKC, JNK, and nuclear factor of activated T cells (NFATs), acting on transcriptional activity [198]. WNT signaling is involved in the regulation of normal hematopoiesis and the specification of hematopoietic differentiation in hematopoietic stem cells [196,198,199].

Studies demonstrated that VDDs exert inhibitory effects on WNT/ $\beta$ -catenin signaling through both direct and indirect mechanisms [200,201]. In colon cancer cells [197], the primary direct inhibitory mechanism is the binding of  $1,25(\text{OH})_2\text{D}_3$ -bound VDR to  $\beta$ -catenin, which inhibits at least some WNT/ $\beta$ -catenin transcriptional activity [202]. The indirect mechanisms involve VDR-regulated gene products; primarily the induction of DKK-1, which binds to LRP5/6 and prevents the initiation of the WNT signaling cascade [200], and also the upregulation of E-cadherin, which binds to  $\beta$ -catenin in the cytoplasm and reduces its translocation into the nucleus [55]. The  $1,25(\text{OH})_2\text{D}_3$  analog calcipotriol was found to target LRP6 through transcriptional upregulation of LDL receptor adaptor protein 1 (LDL-RAP1) to inhibit WNT signaling in pancreatic cancer [203]. Interestingly, it was demonstrated that the nongenomic  $1,25(\text{OH})_2\text{D}_3$  pathway and the noncanonical WNT5a pathway can interact through the shared components of their membrane  $1,25(\text{OH})_2\text{D}_3$  and WNT receptors to regulate the balance between proliferation and differentiation of chondrocytes [204].

The dysregulation of WNT/ $\beta$ -catenin signaling has been shown to contribute to the development of myeloid and lymphocytic leukemias [196]. The products of balanced translocations have been shown to activate the WNT signaling pathway in AML cells [205], and the loss of  $\beta$ -catenin disrupts the expression of multiple oncogenes in AML and reduces the incidence of leukemia cell self-renewal [196,206]. WNT/ $\beta$ -catenin signaling upregulates the transcription of the RUNX1-ETO fusion gene that is created by the reciprocal 8:21 translocation in AML cells and contributes to the neoplastic phenotype [207]. The WNT pathway is also involved in the generation and maintenance of chronic myelogenous leukemia (CML) in vivo [196]. Importantly from a therapeutic perspective,  $1,25(\text{OH})_2\text{D}_3$  was shown to suppress the development of breast cancer through suppressing  $\beta$ -catenin activity [208], and the activation of WNT/ $\beta$ -catenin signaling was shown to be important in the acquisition of drug resistance in acute leukemia cells in the bone marrow microenvironment [209]. Also,  $1,25(\text{OH})_2\text{D}_3$ /VDR signaling was reported to suppress WNT/ $\beta$ -catenin signaling in melanoma, and this was associated with less metastatic disease and stronger host immune responses [210].

## 2.8 JAK/STAT pathway

Characterization of this pathway may lead to advances in the understanding of leukemogenesis and may have potential for improved therapy of hematological neoplasms [158,211]. Particularly, constitutive activation of STAT5, ERK, and AKT in the leukemic stem cell (LSC) population of bone marrow–derived blasts from patients with AML was associated with poor survival and failure to achieve remission [211]. A phase I trial showed that ruxolitinib, a JAK-1 and JAK-2 inhibitor currently approved for patients with severe myelofibrosis, can induce objective responses in patients with chronic myelomonocytic leukemia [212]. It has been shown that  $1,25(\text{OH})_2\text{D}_3$  and its analog EB1089 selectively inhibit IL-2-induced STAT1 and STAT3 phosphorylation and inflammatory cytokine production in NK cell large granular lymphocyte leukemia (NK-LGLL) cells characterized by hyperactivated JAK/STAT pathway [213,214]. However, AML cells with mutations, which lead to constitutive activation of STAT1/STAT5, have low expression of VDR and are not responsive to VDDs [215,216]. On the other hand, a recent study has demonstrated that coordinated activation of the JAK-STAT, p38 MAPK, and NF- $\kappa$ B pathways is necessary for the synergistic upregulation of CYP27B1 expression by IFN $\gamma$  and CD14/TLR4 binding in monocytes. This upregulation correlated with increased  $1\alpha$ -hydroxylase activity and production of  $1,25(\text{OH})_2\text{D}_3$  [217]. Moreover, this and previous studies showed that  $1,25(\text{OH})_2\text{D}_3$  does not downregulate the induced  $1\alpha$ -hydroxylase in monocytes [218]. This is in a striking contrast to kidney cells, in which  $1,25(\text{OH})_2\text{D}_3$  is a negative regulator of CYP27B1 expression [219].

## 3. Transcription factors in VDD-induced differentiation

In addition to VDR, which is directly activated by VDDs and heterodimerizes with a member of RXR family to induce transcription of VDRE-containing genes, several ubiquitous transcription factors also appear to be involved, perhaps in a contributory way, in VDD-induced differentiation (Table 86.2). These may act by interacting with the adjacent VDREs [220,221], by upregulating VDR expression through its promoter region [222–224], by complexing with other transcription factors [221,225,226], and in other ways that remain to be elucidated. On the other hand, some transcription factors [227,228] were found to repress VDR expression and, thus, to attenuate  $1,25(\text{OH})_2\text{D}_3$  signaling. Besides VDR, certain vitamin D metabolites, e.g.,  $20,23(\text{OH})_2\text{D}_3$  or its precursor  $20(\text{OH})\text{D}_3$ , may activate additional ligand-dependent transcription factors, such as aryl

hydrocarbon receptor [229], thereby expanding the repertoire of cellular regulatory pathways affected by VDDs.

## 3.1 Transcription factors involved in developmental myeloid differentiation

During differentiation of HSPCs, specific transcription factors are expressed at crucial developmental stages and regulate, positively or negatively, myeloid commitment of these precursors in both health and various diseases. Among the key regulators of early hematopoiesis are runt-related transcription factor 1 (RUNX1; also known as AML1), ETS variant transcription factor 2 (ETV2), and stem-cell leukemia factor (SCL; also known as TAL1) [230]. RUNX1 is essential for the formation of HSCs and hematopoietic progenitors via facilitated expression of critical regulators of the endothelial-to-hematopoietic transition (EHT), such as growth factor independent 1 (GFI1) and GFI1B [231]. The upregulation of RUNX1 during the EHT is responsible for the upregulation of PU.1/SFPI1, a master regulator of myelopoiesis and B cell development [231,232]. Following PU.1, other transcription factors are upregulated during myelopoiesis, including CCAAT/enhancer binding protein (C/EBP) $\alpha$ , followed by C/EBP $\beta$  and C/EBP $\epsilon$ , growth factor independent 1 (GFI1) and interferon-regulatory factor 8 (IRF8; also known as ICSBP). Macrophage differentiation depends on PU.1 and IRF8. GFI1, C/EBP $\alpha$  and C/EBP $\epsilon$  are crucial for neutrophil differentiation. On the other hand, IRF8 blocks C/EBP $\alpha$  activity to suppress the neutrophil differentiation program [233]. The aforementioned transcription factors regulate the expression of many myeloid genes, such as those encoding receptors for M-CSF, G-CSF, and GM-CSF [234,235]. GATA-1 is induced by IL-4 during dendritic cells differentiation from monocytes [227].

Aged human HSCs have been shown to have myeloid-biased differentiation potential compared with young HSCs. This was accompanied by upregulation of genes associated with cell cycle, myeloid lineage specification, and myeloid malignancies [236]. Particularly, dysregulation of the monocyte/macrophage differentiation primary response transcription factor EGR-1 in aged HSCs was linked to the reduction of G2/M phase of the cell cycle and induction of quiescence regulators, such as JUNB, BTG antiproliferation factor 2 (BTG2), and nuclear receptor subfamily 4 group A member 1 (NR4A1) [237]. Chronic infection also results in increased myeloid differentiation of HSPCs, which leads to their depletion in the bone marrow. This differentiation effect was found to be induced by accumulated IFN $\gamma$  through activating the



basic leucine zipper ATF-like transcription factor 2 (Batf2) [238].

Several negative regulators of myeloid differentiation have been identified. For instance, the RNA-binding protein ZFP36L2 was found to interact with the 3' untranslated region of key myeloid maturation genes to promote their mRNA degradation and suppress terminal myeloid cell differentiation. Conversely, genetic inhibition of ZFP36L2 led to upregulation of several key monocytic/macrophage-associated genes (e.g., CD14, CSF1R, CEBPB, IL1B, and MMP9) and downregulation of genes associated with leukemia stem cell signatures [239]. Another example is the homeobox transcription factor HB9 whose aberrant expression is a molecular hallmark of infant t(7;12) (q36;p13) AML [240]. HB9-transduced HSPCs underwent a profound differentiation arrest and accumulated at the megakaryocyte/erythrocyte progenitor stage in vivo [241]. High expression of Iroquois homeobox transcription factor 3 (IRX3) was shown to be associated with reduced myelomonocytic differentiation, and IRX3 knockdown induced terminal differentiation of AML cells [242]. Expression of mutant RUNX1 was found to enhance self-renewal and impair myeloid commitment of HSPCs. CEBP $\alpha$  expression was reduced in RUNX1 mutant-expressing cells, and reexpression of CEBP $\alpha$  partly restored differentiation [243]. In patient-derived AML blasts, increased occupancy of CTCF-binding factor (CTCF), which regulates gene expression through chromatin organization, was found in DNA motifs for key myeloid transcription factors such as C/EBP $\alpha$ , PU.1, and RUNX1, and CTCF knockdown resulted in increased myeloid differentiation [244]. Leukemia-associated mutant cohesin complex proteins can also impair HSPC differentiation by controlling chromatin accessibility for key differentiation-related transcription factors [245]. Expression of the AML-associated chimeric transcription factor RUNX1-ETO in normal myeloid progenitor cells was shown to interfere with RUNX1 binding, leading to extensive chromatin reprogramming and differentiation block [246].

### 3.1.1 Activator protein-1 transcription factor

ERK and JNK pathways activate members of the FOS and JUN families, as well as some related proteins, which dimerize in various combinations to form the AP-1 transcription factor [247,248]. Thus, AP-1 can integrate and transmit signals transduced by the MAPK pathways previously discussed. Integrated analysis of epigenetic profiles, gene expression, and chromatin conformation data in purified leukemic blasts from multiple AML samples has shown that mutated components of the MAPK/AP-1 pathway form a prominent regulatory network in most of the AML subsets. Notably, the

expression of a dominant negative form of c-FOS in AML cells carrying t(8;21) translocation (Kasumi-1) and FLT3-ITD mutation (MV4-11) was sufficient to block cell growth in vitro and tumor formation in mice [249,250].

Several studies have shown that the expression of c-JUN is increased during the 1,25(OH) $_2$ D $_3$ -induced differentiation of human myeloid cells [156,251,252] and coordinates the occupancy of AP-1 sites and VDRE elements by their cognate transcription factors. This can provide a possible model for the reciprocal relationships between different cellular phenotypes and functional activities, such as those that occur during differentiation. For example, there is also evidence for functional cooperation between VDR and RAS-activated ETS transcription factors in 1,25(OH) $_2$ D $_3$ -mediated induction of the VDD-metabolizing enzyme 24-hydroxylase (CYP24A1) gene expression [253]. Liu and Freedman [220] conducted an extensive study of such transcriptional synergism between VDR and nonreceptor transcription factors and concluded that the functional basis for such synergism appears to be at the level of cooperative DNA binding.

AP-1 activation by 1,25(OH) $_2$ D $_3$  has been described in diverse differentiation systems. These include HL60 cells [156,254], colon cancer CaCo-2 cells [255], osteoblasts [256], keratinocytes [257], and breast cancer cells [142]. Interestingly, the pathways that signal AP-1 activation are apparently cell type and cell context specific. For instance, the p38 and JNK MAPK pathways cooperate to activate VDR by c-JUN/AP-1 in breast cancer cells [142], while in keratinocytes and HL60 leukemia cells, AP-1 activation is attributed to both the ERK and JNK pathways, e.g., in the presence of plant antioxidants [156,258,259].

The AP-1 complex has variable components, some of which can interact with the VDR transcriptional machinery. For instance, the exposure of the CML cell line RWLeu-4 to 1,25(OH) $_2$ D $_3$  inhibited their proliferation and enhanced the binding activity of the proto-oncogene JUN-D to the VDRE [260]. Exposure of HL60 cells to 1,25(OH) $_2$ D $_3$  upregulated expression of genes that code for the AP-1 complex including c-JUN, ATF-2, JUN-B, and FOS-B [156,261]. Moreover, 1,25(OH) $_2$ D $_3$  (10 $^{-7}$  M) induced within 6 h the expression of the subunits of the transcriptional coactivator, vitamin D receptor-interacting proteins (DRIP, also called thyroid hormone receptor-associated polypeptide; TRAP), in HL60 cells [262]. The DRIP complex plays a role in direct communication between the nuclear receptors and the general transcriptional machinery through direct interaction with RNA polymerase II [263]. DRIP knockdown-HL60 cells as well as murine TRAP220 $^{-/-}$  yolk sac hematopoietic progenitor cells are resistant to induction of differentiation by 1,25(OH) $_2$ D $_3$ .



Thus, the aforementioned examples show that AP-1 transcription factor appears to be an important integrator of converging differentiation pathways.

### 3.1.2 Specificity protein 1 transcription factor

The SP1, a 95–105 kDa protein, is ubiquitously expressed in growing cells, and, usually in combination with other factors, acts as a transcriptional activator of many housekeeping genes [264–269]. Its role in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation has been suggested in human myeloid leukemia cells. Specifically, the DNA binding of SP1 was found to be increased following an exposure of HL60 cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> [254,270], while in U937 cells, SP1 may participate in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced expression of CD14 monocyte marker, which has several SP1-binding sites in its promoter [271], as well as the CD11b promoter [272]. It was also shown that upregulation of p27<sup>Kip1</sup> in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated U937 can be mediated by SP1 [273], as discussed in the following.

### 3.1.3 Master transcription factors

1,25(OH)<sub>2</sub>D<sub>3</sub> can induce master transcription factors of myeloid differentiation to regulate gene expression. For example, exposure of AML cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> induced the expression of PU.1 and IRF8 (e.g., Ref. [274]) but, in contrast, downregulated the expression of ETV7 (also known as TEL2), which is a member of the ETS family [275]. Interestingly, forced overexpression of ETV7 inhibited 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation. PU.1 knockdown has been shown to drive AML development, while its restoration triggers AML differentiation and clearance in vivo [276]. Notably, PU.1 suppression in differentiating AML cells can induce dedifferentiation and reacquisition of clonogenic and leukemogenic properties [276].

The activation of the master transcription factor, proto-oncogene c-MYC, is a typical feature of human leukemias and lymphomas (reviewed in Refs. [277,278]). The HL60 leukemia cell line is characterized by high levels of expression of c-MYC due to gene amplification [162,279]. Treatment of these cells with 1,25(OH)<sub>2</sub>D<sub>3</sub> results in a downregulation of expression of c-MYC associated with the induction of cell differentiation [280–282]. This suppression of c-MYC occurs at the transcriptional level [281–283]. A similar c-MYC downregulation was observed in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated head and neck squamous cell carcinoma (HNSCC) cells and primary keratinocytes, while the expression of the c-MYC antagonist MAD1/MXD1 was enhanced [282]. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> induced a rapid association of the VDR with c-MYC, suggesting that 1,25(OH)<sub>2</sub>D<sub>3</sub> may also control c-MYC stability [282]. 1,25(OH)<sub>2</sub>D<sub>3</sub> also upregulates the protein encoded by the homeobox gene HOXB4 that binds to the first exon/intron border

of c-MYC to prevent transcriptional elongation, a process dependent on activation of PKC-β [286]. Another homeobox gene, HOXA10, was found to be transcriptionally induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> through binding to the VDRE in the promoter during differentiation of U937 cells [285].

Exposure of HL60 cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of the proto-oncogene c-FMS, which encodes the receptor for M-CSF. It occurs in parallel with the induction of CD14 expression and a block of the cell cycle in the G0/G1 phase [286]. cDNA microarray analysis showed that at early times of VDD exposure, the putative oncogenes DEK and FLI1 were downregulated and the antiproliferative gene BTG1 was upregulated.

### 3.1.4 Other transcription factors that contribute to VDD-induced differentiation

Other transcription factors can be induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> in differentiating myeloid cells. For instance, high expression levels of PPARδ are detected in the AML cells with an AML-M5 (monoblastic) phenotype. Furthermore, the forced overexpression of PPARδ suppressed the 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated monocyte–macrophage differentiation [287]. Upregulation of PPARδ expression was found upon overexpression of the H2.0-like homeobox transcription factor (HLX) in zebrafish and human HSPCs. Pharmacological inhibition of PPARδ signaling relieved the HLX-induced myeloid differentiation block [288].

Increased expression of monocytic lineage transcription factor EGR-1 by 1,25(OH)<sub>2</sub>D<sub>3</sub> in myeloblastic HL60 cells (AML-M2) was shown to contribute to onset of the terminal phase of monocyte/macrophage differentiation [289]. Interestingly, EGR-1 levels were also increased by 1,25(OH)<sub>2</sub>D<sub>3</sub> in granulocyte-committed acute promyelocytic leukemia (APL; AML-M3) NB4 cells. Furthermore, GFI1, which promotes the granulocytic lineage, was upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> in HL60 cells and by ATRA in myelomonocytic (AML-M4/M5) U937 cells [290].

It has been documented that retinoic acid receptor α (RARα) is able to regulate expression of VDR in AML cells [224], and to much lower extent in normal human blood cells [291]. This regulation is quite complex, not fully understood, and depends on the levels of RARα protein in the cells. In cells with high levels of RARα protein, this unligated transcription factor inhibits the expression of VDR, while it upregulates VDR expression upon binding the ligand, ATRA. Interestingly, the regulation is opposite in those AML cells that have low levels of RARα protein [137,224].

Fusion proteins involving the RARα with either the PML or PLZF nuclear proteins can provide genetic markers of APLs. APL cells expressing PML-RARα fusion protein are sensitive to retinoid-induced

differentiation to granulocytes in the presence of ATRA. In contrast, forced expression of either PML-RAR $\alpha$  or PLZF-RAR $\alpha$  in either U937 or HL60 cells blocked their terminal differentiation after exposure to 1,25(OH) $_2$ D $_3$  [292]. Both PML-RAR $\alpha$  and PLZF-RAR $\alpha$  can bind to VDR in U937 cells and sequester VDR away from activation of its normal DNA targets [150]. Overexpression of VDR overcomes the block in 1,25(OH) $_2$ D $_3$ -stimulated differentiation caused by the fusion proteins. Of note, PLZF itself can interact directly with VDR, and overexpression of PLZF can inhibit the 1,25(OH) $_2$ D $_3$ -induced differentiation of U937 cells [149].

Undoubtedly, many other transcription factors, including C/EBP $\beta$ , contribute to 1,25(OH) $_2$ D $_3$ -induced differentiation of AML cells [128,293,294]. It has been reported that C/EBP $\beta$  isoforms are activated by phosphorylation by ERK [295] and by RSK [296], and interact directly with the promoter of one of the principal markers of monocytic differentiation - CD14 [297]. In myeloid leukemia cells, hematopoietic progenitors are unable to undergo granulocytic differentiation, as a result of various mutations, which alter the proper balance of transcription factor activity. Elevated expression of C/EBP $\beta$  induced by 1,25(OH) $_2$ D $_3$  allows the cells to bypass this block by switching the lineage of differentiation to monocyte-like cells instead of granulocytes [294].

Other transcription factors may also contribute to the eventual cell cycle arrest, and some of these will be discussed relative to cell cycle control. The important distinction between the transcription factors, which regulate the expression of new genetic programs, and those that control functions of the differentiated cells, is not easy to make at present.

## 4. Effects of VDDs on the cell cycle and proliferation of human leukemia cells

### 4.1 General features of the cell cycle

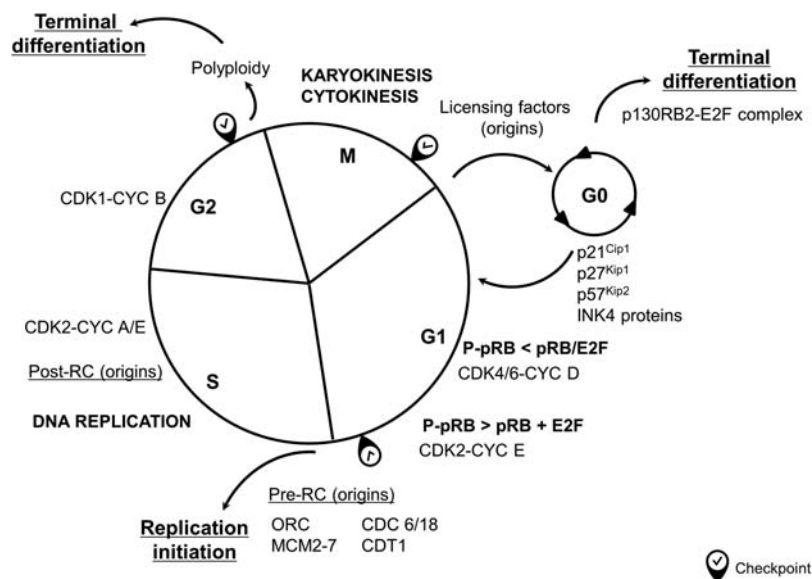
In general, the cell cycle and its regulation are extremely well conserved throughout the eukaryotic species. The consecutive progression through the G1, S, G2, and M cell cycle phases results in proliferation of eukaryotic cells (Fig. 86.3). DNA replication occurs during the S phase; chromosome separation (karyokinesis) takes place during the M phase and is followed by cell division (cytokinesis); G1 and G2 are gap or growth phases when molecules required for DNA replication or mitosis are synthesized. Cells that are not actively dividing may either be permanently removed from these cycling phases by terminal differentiation, senescence or apoptosis, or be temporarily arrested in a noncycling quiescent state known as G0 if the cells

have the G1 DNA content [298]. The basic cell cycle machinery consists of several cyclin-dependent kinases (CDKs) and cyclins that pair with each other, sometimes changing partners, to drive the cell toward and through mitosis [299,300]. CDKs are usually present throughout the cell cycle, while cyclin levels oscillate in a cell cycle-dependent manner. This basic arrangement occurs in all cells, despite the necessity for cell type specificity of proliferation control in multicellular organisms. The most studied target of cyclin/CDK activity is the retinoblastoma susceptibility protein (pRB); the phosphorylation of pRB is the principal mechanism regulating cell transit through G1.

Control of cell cycle transit is provided primarily by the regulation of cyclin/CDK activities, which occur in response to various internal and external cues. Importantly, cell cycle changes are also triggered in cells undergoing differentiation. A series of checkpoints control the traverse of the various cell cycle compartments [301]. Checkpoints may be simply defined as mechanisms that prevent cell cycle progression until a specific requirement has been met. Checkpoints operate in each phase of the cell cycle: pRB phosphorylation acts as a G1 checkpoint. Checkpoints control each phase of the cell cycle. pRB phosphorylation acts as a G1 checkpoint [302], and the CHK1 and CHK2 kinases act as S phase checkpoints in response to DNA damage [303]. Other regulators such as p53 have been shown to have importance in checkpoint control, though in hematopoietic malignancies, p53 is often inactivated by mutations. Interestingly, these regulators of cell cycle progression may also influence cellular decisions to differentiate in embryonic, normal adult, and transformed phenotypes [303,304]. The final result of the cell cycle traverse is usually a faithful replication and accurate partitioning of genetic information.

Differentiation can be considered to be, in essence, a persistent change in the pattern of previously expressed genes, which results in new functional capabilities of the differentiated cell. The new functions require cellular resources that compete with, and finally titrate out the resources required for proliferation, and allow an accumulation of negative regulators of the cell cycle (e.g., p27<sup>Kip1</sup>), which eventually predominate over the positive regulators. Thus, there is a reciprocal relationship between cellular differentiation and cell cycle progression/proliferation [305], though there is also evidence that differentiation and cycle arrest need not be strictly coupled [31,306,307].

Cell cycle changes in differentiating cells need not take place immediately - in some cells there is at first a boost of proliferation - as in normal hematopoiesis, or in HL60 [183,307] and U937 [153] cells differentiating in response to VDDs. In at least some of these cells, the initial burst of proliferation following the introduction



**FIGURE 86.3** The general concept of the cell cycle, and examples of factors that control cell cycle traverse and DNA replication. In most cells, the principal locus for terminal differentiation is in G0 phase, but it can also occur in G2, associated with polyploidy. In early G1, the level of phosphorylated retinoblastoma protein (P-pRB) is lower ( $<$ ) than the level of hypophosphorylated pRB protein complexed with transcription factors of the E2F family (pRB/E2F). Phosphorylation of pRB by CDK4/6-CYC D and later by CDK2-CYC E results in high levels of P-pRB, dissociation of P-pRB from the E2Fs, and transcription of E2F-regulated genes, allowing passage through the restriction point (fx1). Replication origins are bound by licensing factors in late M/early G1, creating replication-competent origins for use in S phase. Additional details of the controls of cell cycle progression are provided in the text and illustrated in Fig. 86.4. RC, replicative complex; ORC, origin recognition complex; MCM, minichromosome maintenance complex; licensing factors, ORC, MCM, and other components of the pre-RC complex; CYC, cyclin.

of the differentiating agent is not only concomitant with the initiation of differentiation, but it is required - if proliferation in those cells is inhibited when the differentiating agent is added, the differentiation process is halted [299,305]. However, even in these cases, there is an eventual slowdown of the cell cycle traverse and cessation of proliferation of differentiated cells. Consequently, numerous attempts are being made to exploit the differentiating actions of  $1,25(\text{OH})_2\text{D}_3$  and its analogs to induce proliferative quiescence of neoplastic cells, and thus increase the range of options for optimal therapy of human cancer [308].

## 4.2 Regulation of cell cycle progression

### 4.2.1 Progression through G1 and G1/S boundary

The primary drivers of G1 cell cycle transit are the kinase activities of cyclin/CDK complexes, which are controlled by a number of different mechanisms. The kinase activities of these complexes are tightly regulated by multiple mechanisms, including cyclin binding to CDKs, cyclin synthesis, cyclin degradation, cyclin/CDK phosphorylation and dephosphorylation at several key sites, and interactions with several classes of CDK inhibitors. CDKs are present throughout the cell cycle but are active only in complexes with the appropriate cyclin; each class of cyclins is synthesized and degraded

at different and specific points in the cell cycle. Mitogenic signals that stimulate cell progress through G1 increase the expression of cyclin D isoforms in early G1, which form complexes with CDK4 and/or CDK6. Later in G1, cyclin E is synthesized, peaking at the late G1/S phase boundary. The expression of cyclin E is mitogen independent, and cyclin E forms an active complex with CDK2 (Fig. 86.3). The activity of cyclin-CDK complexes depends on both activating and inhibitory phosphorylations. CDK1 and CDK2 are activated by CAK-mediated phosphorylation of Thr160/161 and inhibited by phosphorylation of Thr14 and Tyr15 by WEE1 and MIK1. Removal of Thr14 and Tyr15 phosphate groups by the CDC25A, B, and C phosphatases activates the cyclin/CDK complex. These protein phosphatases are linked to cell cycle checkpoint mechanisms, as the checkpoint kinases CHK1 and CHK2 can both phosphorylate CDC25A and target it for destruction [309–311].

Another level of CDKs regulation is provided by the CDK inhibitory proteins (CKIs), which prevent CDK activation by binding to the kinases and thus preventing their activation by cyclins. There are two major CKI families. One is the INK4 family, which includes CDKN2A (p16-INK4A), CDKN2B (p15-INK4B), CDKN2C (p18-INK4C), and CDKN2D (p19-INK4D) [312–314]. The other is the Cip/Kip family, whose main members are CDKN1A ( $p21^{Cip1}$ ) and CDKN1B

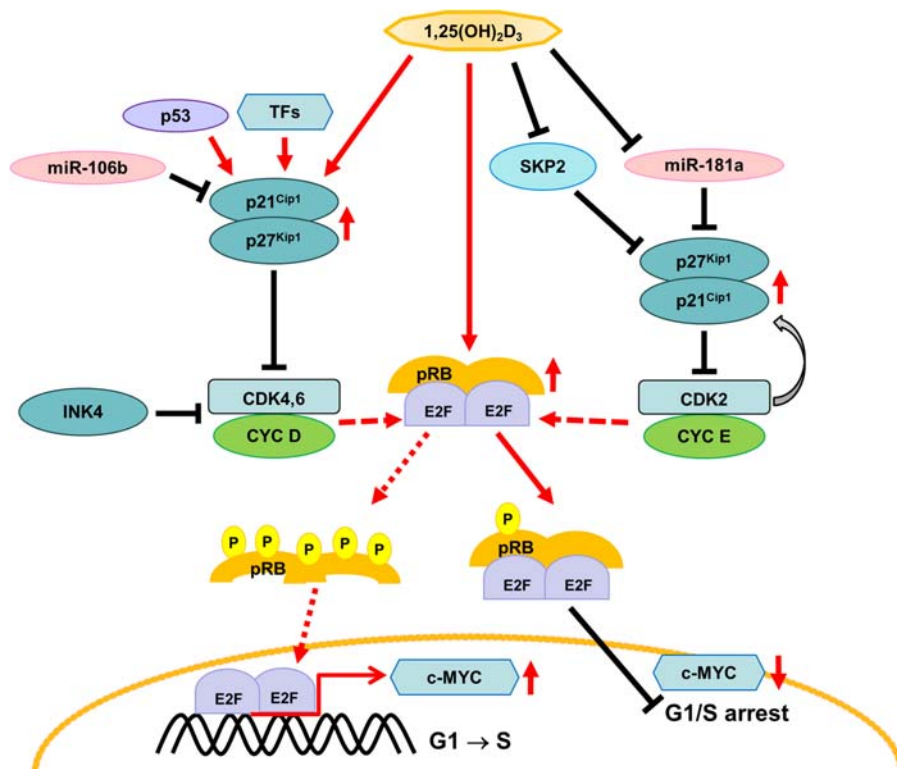
(p27<sup>Kip1</sup>). The INK4 proteins specifically block cyclin D-CDK4/6 activity leading to a G1 phase arrest, both by preventing the binding of cyclin D and by inhibition of formed CDK4/6-cyclin D complexes [315,316]. The Cip/Kip proteins are elevated during periods of both proliferation and growth inhibition [317,318] and are upregulated in a time-dependent manner after exposure of cells to differentiating agents, such as U937 cells exposed to 1,25(OH)<sub>2</sub>D<sub>3</sub> [152]. The Cip/Kip family members p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup> are well-established inhibitors of cyclin D-CDK4/6 and cyclin E-CDK2 activities and exert significant control over the entry into the S phase [317] (Fig. 86.3). All three of these inhibitors block progression through the G1 phase but are usually activated by different stimuli. The expression of p21<sup>Cip1</sup> can be under the transcriptional control of the p53 tumor suppressor gene, activated by DNA damage [319], but may also be independent of p53 [320,321], including regulation by microRNAs [322], or 1,25(OH)<sub>2</sub>D<sub>3</sub>-activated VDR [152]. p27<sup>Kip1</sup> participates in G1 arrest produced by external stimuli such as cell–cell contact or differentiating agents as discussed in the following. While there

is a large body of evidence showing the regulation of Cip/Kip CDKIs by 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs (see the following), the control of INK4 proteins by VDDs has not been established.

The principal target of cyclin/CDK activity is the tumor suppressor pRB (Figs. 86.3 and 86.4). The phosphorylation of pRB, and other pRB-like “pocket” proteins (p130/RB2, p107) control the entry into the S phase by interacting with a member of the E2F transcription factors family. In its simplest form, the current hypothesis is that hypophosphorylated pRB binds to E2Fs and blocks their transcriptional activity; upon increased level of phosphorylation, pRB dissociates from E2F, allowing the transcription of genes necessary for S phase initiation, such as c-MYC/MAX. However, the actual situation appears to be more complicated since gene repression by pRB also involves modulation of chromatin architecture by epigenetic mechanisms [323].

#### 4.2.2 Control of S phase and DNA replication licensing

DNA replication occurs in S phase, and the basic machinery for this process is well conserved from



**FIGURE 86.4** Control of G1 to S phase transition and G1/S arrest by the pRB-E2F pathway: Regulation of Cip/Kip family CDKIs by VDDs. Phosphorylation of pRB by active CDKs releases E2F transcription factors, which activate, directly or indirectly, genes whose products are required for DNA replication [663,664]. The activity of CDKs can be controlled by multiple mechanisms including transcriptional regulation, cyclin binding, activating and inhibitory phosphorylations, and CDK inhibitor (CDKI) binding. CDKIs belong to two major families, the Cip/Kip family and the INK4 family. VDDs upregulate both p21<sup>Cip1</sup> and p27<sup>Kip1</sup> in temporally distinct patterns, which inhibit CDK/cyclin activities and G1 transit. c-MYC is one of the key genes controlled by E2Fs that regulates over 1600 known target genes.



yeast to mammals (reviewed in Ref. [324]). Replication in mammalian cells is initiated on many sites on chromosomes, designated as “replication origins,” which can exist in two states. In G1 phase, a multiprotein complex, the prereplicative complex (pre-RC), is assembled, at which time the origin is considered to be “licensed” and the chromatin becomes competent for DNA replication. Components of the pre-RC, the “initiator” proteins, include the origin recognition complex (ORC), CDC6/18, CDT1, geminin, and minichromosome maintenance 2–7 (MCM 2–7) proteins. The six MCM proteins form a hexameric complex, which may function as a replicative helicase. Once DNA replication is initiated, the complex has fewer components of the postreplicative complex (post-RC) and persists to the end of mitosis, functioning along with other molecular mechanisms, to block rereplication of DNA. At that time point, proteolytic activity destroys the cyclins and other nuclear proteins, and CDK activity becomes low. These two states of replication origins, separated by CDK activity, generally ensure that the S phase and mitosis alternate. Licensing in G1 phase is permitted after the end of mitosis, when geminin is destroyed by the anaphase promoting complex (APC)–ubiquitin system [325]. The key steps in this sequence are illustrated in Fig. 86.3.

In the higher multicellular eukaryotes but not in yeast, chromatin–ORC interactions are dynamic with ORC subunits cycling on and off replication initiation sites, which allows the changes in initiation site location that occur during differentiation [326]. When the cells exit mitosis, CDC6/18 and CDT1 are loaded on chromatin, and in turn aid loading of MCM on the pre-RC complex, thus completing licensing. The licensed complex can now be activated for DNA replication by a protein kinase, such as cyclin E/CDK2 or DBF4-dependent kinase, and the DNA replicating machinery (e.g., CDC45, replication protein A, DNA polymerase  $\alpha$  and  $\epsilon$ ) is recruited to the initiation sites.

Sites of replication in mammalian cells also change with the alterations of chromosomal architecture caused by modulation of gene activity that occurs during differentiation. Replication origin sites correlate with sites of transcriptional units, the sites of ORC1-binding correlate with transcriptional start sites, and transcription at those sites correlates with replication timing [327]. A number of pre-RC proteins are known to associate with transcription factors, including most of those implicated in stem cell maintenance and development (including c-MYC, OCT4, SOX2, Nanog, and HOXD-13), suggesting that at least part of stem cell programming and development involves the regulation of the positioning and activity of replication origins in chromatin [328].

#### 4.2.3 The G2 and M phase transition

In the G2 and M phases, cells synthesize mitotic components and segregate the duplicated DNA into equivalent, or nearly equivalent, daughter cells (reviewed in Ref. [329]). The central regulator of G2/M passage is the cyclin B–CDK1 complex. The activity of this complex is governed by factors similar to those responsible for the G1/S transition, including phosphorylation by CAK and dephosphorylation by CDC25A, CDC25B, and CDC25C. The correct segregation of duplicated chromatin into the two daughter cells requires two additional levels of regulation of proteins that bind together the two chromatids, and control of centrosome duplication and spindle assembly.

The sister chromatids adhere to one another by the adhesive properties of a multisubunit complex called “cohesin” [330]. Cohesin is dissolved by proteolytic cleavage of one of its subunits, SCC1/MED1, by separase, a calcium-activated cysteine protease [331]. The dissolution of cohesion between the chromatids occurs in two steps, one at prophase, the other at anaphase, and only the latter requires separase. Separase is regulated by cyclin B-CDK1 phosphorylation and by the inhibitor securing. Both cyclin B and securin are ubiquitinated by the APC and destroyed at the end of mitosis, thus ensuring orderly and precisely timed separation of the chromosomes at telophase [332]. Polo-like kinase-1 (PLK1) also regulates chromosome adhesion and other aspects of mitosis, including centrosome maturation and orientation. PLK1 has been reported to phosphorylate cyclin B1 and target it to the nucleus during prophase, and the finding of colocalization of PLK1 and CHK2 suggests that there is a lateral communication between the mitotic checkpoint and the DNA integrity [333,334]. Moreover, a molecular reciprocal activation between PLK1 and N-MYC was reported, which leads, among other factors, to the reduced degradation of N-MYC, cyclin E, and Mcl1 and reinforces MYC-regulated oncogenic programs [335]. Recently, it was also reported that in NSCLC cells, the transcription factor C/EBP $\beta$  directly represses Wee1 expression, which is required for the G2/M phase progression [336].

#### 4.3 Modulation of cell cycle events by vitamin D and its analogs

The changes in cell cycle traverse and DNA replication that occur in numerous forms of differentiation have been previously reviewed [305]. Changes that specifically follow exposure to VDDs have been less extensively studied, and these will now be described. The inhibition of cell cycle traverse by 1,25(OH) $_2$ D $_3$  and other VDDs has been investigated in normal and

malignant keratinocytes [337,338], and in many other types of tumor cells [339–341], with myeloid leukemia cells providing an excellent in vitro model system for this purpose [140,342,343]. Leukemic cells show complex and time-dependent responses to  $1,25(\text{OH})_2\text{D}_3$ ; for example, myeloid leukemia cell lines cultured with  $1,25(\text{OH})_2\text{D}_3$  undergo an initial proliferative burst, which is followed by growth inhibition, terminal differentiation, and subsequent apoptosis [153,344]. In U937 myelomonoblastic leukemia cells, levels of cyclin A1, D1, and E increase within 24 h of  $1,25(\text{OH})_2\text{D}_3$  treatment and then expression decreases after 48 h, a change in expression that parallels the change in cell proliferation following  $1,25(\text{OH})_2\text{D}_3$  treatment [153].

#### 4.3.1 The G1/S block

$1,25(\text{OH})_2\text{D}_3$  and its analogs inhibit proliferation of diverse types of mammalian cells by arresting them in the G1/G0 phase of the cell cycle. While the exact sequence of events from VDR activation to G1/G0 arrest remains to be elucidated and may not be exactly the same in all cell types, several pathways and cell cycle arrest effectors are already known to be involved. These include the upregulation of protein levels of the CDK inhibitors  $p21^{\text{Cip1}}$  and/or  $p27^{\text{Kip1}}$ , the upregulation of RB gene expression and phosphorylation of the pRB protein, and the inhibition of c-MYC expression [37,280,345–348].

#### 4.3.2 Upregulation of $p21^{\text{Cip1}}$ and $p27^{\text{Kip1}}$

Elevated protein levels of the Cip/Kip family of CDKIs result from the exposure to VDDs in many cell types including hematopoietic cells (Table 86.3) and may be a near-universal feature of the antiproliferative effects of these compounds. In view of the importance of CDK activity as the driving force for cell cycle progression, it is not difficult to understand that increased levels of CDKIs can target CDK complexed to cyclin D, E, or A, and grind the cell cycle traverse to a halt. However, the mechanisms of the CDKI upregulation are not entirely clear, and there are subtle differences between the antiproliferative effects of  $p21^{\text{Cip1}}$  and  $p27^{\text{Kip1}}$ . In contrast,  $p57^{\text{Kip2}}$  was seldom found to have a role in the antiproliferative effects of vitamin D and its analogs, apart from the possible involvement in the transit of osteoblasts from proliferation to differentiation [349].

In U937 myelomonocytic leukemia cells, a correlation was reported between early induction of  $p21^{\text{Cip1}}$  and the beginning of the differentiation program. The upregulation of  $p21^{\text{Cip1}}$  mRNA occurred within 4 h of the exposure to  $1,25(\text{OH})_2\text{D}_3$  independent of de novo protein synthesis, suggesting a direct transcriptional activation by VDR [152]. Indeed, the  $p21^{\text{Cip1}}$  promoter contains VDREs, and induction requires the presence of VDR. Nevertheless, some data have suggested that the

marked increase of  $p21^{\text{Cip1}}$  protein expression in response to  $1,25(\text{OH})_2\text{D}_3$  may also be due to enhanced posttranscriptional stabilization of  $p21^{\text{Cip1}}$  mRNA [350]. The transcription factor p53 is a strong inducer of  $p21^{\text{Cip1}}$ , but  $1,25(\text{OH})_2\text{D}_3$  can elevate  $p21^{\text{Cip1}}$  levels independently of p53 activity [346,351–353]. In U937, levels of cyclin A1, D1, and E increase within 24 h of  $1,25(\text{OH})_2\text{D}_3$  treatment, and then expression decreases after 48 h [153]. A strong upregulation of  $p27^{\text{Kip1}}$  protein expression was evident after 72 h exposure of HL60 cells to  $1,25(\text{OH})_2\text{D}_3$ , and the levels of the protein were dependent on the concentration of this compound [346]. This upregulation was also associated with increased levels of cyclin D1 and E, coinciding with a G1 arrest. These results suggested a prominent role of  $p27^{\text{Kip1}}$  in mediating the antiproliferative activity of  $1,25(\text{OH})_2\text{D}_3$  in this cell line. However, some redundancy of cell cycle control is likely and is exemplified by a recent report that  $p27^{\text{Kip1}}$ , CDK and E2F transcriptional repression by the pRB can have interchangeable roles in cell cycle control and tumor suppression [354]. This showed that pRB can control cell cycle progression independently of E2F.

Considerable excitement was generated when  $p21^{\text{Cip1}}$  was found to be upregulated in a number of differentiation systems, including HL60 cells and squamous cell carcinomas (SCCs) treated with  $1,25(\text{OH})_2\text{D}_3$  [320,355] (see also Fig. 86.4 and Table 86.3). It was suggested that  $p21^{\text{Cip1}}$ , and/or  $p27^{\text{Kip1}}$ , not only promote the G1 arrest but also contribute to differentiation [356]. It appears, however, that while these CDKIs may not be solely responsible for the G1 block, the data regarding their role in differentiation are conflicting. For instance, mice lacking  $p21^{\text{Cip1}}$  undergo normal development [357], even though  $p21^{\text{Cip1-/-}}$  embryonic fibroblasts show impaired arrest in G1 in response to DNA damage. An imbalance between growth and differentiation can be demonstrated in these cells, and in other in vitro cell differentiation systems with  $p21^{\text{Cip1}}$  knockouts. Keratinocytes, which are  $p21^{\text{Cip1-/-}}$ , and to a lesser extent those with  $p27^{\text{Kip1}}$  knockouts, have an increased proliferative potential [358]. With regard to differentiation, however, Harvat et al. [359] showed that growth arrest resulting from overexpression of  $p21^{\text{Cip1}}$  in mouse primary keratinocytes is not sufficient to induce the expression of markers of differentiation. Further, in malignant counterparts of these cells, the SCC cells, not even growth arrest, are clearly linked to  $p21^{\text{Cip1}}$ , as  $1,25(\text{OH})_2\text{D}_3$  inhibited growth but reduced  $p21^{\text{Cip1}}$  levels in vitro and in SCC tumors [360].

In another system, the myelomonocytic cell line U937, Freedman's group noted transcriptional activation of the  $p21^{\text{Cip1}}$  gene by  $1,25(\text{OH})_2\text{D}_3$  and suggested that this is

**TABLE 86.3** Examples of upregulation of CDKI levels by 1,25(OH)<sub>2</sub>D<sub>3</sub> and other VDDs in hematopoietic cells.

CDKI	Cell type	Functional effect/Comment	References
p21 <sup>Cip1</sup>	HL60	Rapid early gene induction during monocytic differentiation/G1 arrest	[320,350,355,397]
p21 <sup>Cip1</sup>	U937	Transcriptional activation of p21 <sup>Cip1</sup> induces monocytic differentiation	[152]
p21 <sup>Cip1</sup>	U937	Cytoplasmic localization/antiapoptotic effect	[398]
p21 <sup>Cip1</sup>	U937	Antisense to p21 <sup>Cip1</sup> decreases differentiation	[399]
p21 <sup>Cip1</sup>	THP-1	Upregulation of CDKN1A (encodes p21 <sup>Cip1</sup> ) involves three VDR-binding sites	[400]
p27 <sup>Kip1</sup>	HL60, U937, NB4	Proliferation block/G1 arrest	[46,346,347,401]
p27 <sup>Kip1</sup>	HL60	Antisense to p27 <sup>Kip1</sup> reverses G1 arrest	[342]
p27 <sup>Kip1</sup>	HL60	miR-181a abrogates p27 <sup>Kip1</sup> expression and inhibits G1 arrest and differentiation	[20]
p27 <sup>Kip1</sup>	U937	VDR-independent upregulation of p27 <sup>Kip1</sup> gene	[273]
p27 <sup>Kip1</sup>	UF-1	Stabilization of p27 <sup>Kip1</sup> protein	[319]
p27 <sup>Kip1</sup>	B cells, primary	Inhibition of proliferation and differentiation of activated B cells/apoptosis	[402]
p21 <sup>Cip1</sup> /p27 <sup>Kip1</sup>	HL60	Changes in CDK4 activity/G1 arrest	[403]
p21 <sup>Cip1</sup> /p27 <sup>Kip1</sup>	U937	Early proliferative burst followed by growth arrest and differentiation	[153]
p21 <sup>Cip1</sup> /p27 <sup>Kip1</sup>	UF-1	Granulocytic differentiation/proliferation block/G1 arrest	[372]
p21 <sup>Cip1</sup> /p27 <sup>Kip1</sup>	HL60, OCI-AML3	p53-independent regulation of CDKIs expression	[404]
p21 <sup>Cip1</sup> /p27 <sup>Kip1</sup>	K562	Proliferation inhibited by G0/G1 block	[405]

linked to differentiation of these leukemia cells [152]. Importantly, they identified a functional VDRE in the promoter of the p21<sup>Cip1</sup> gene and noted that the p21<sup>Cip1</sup> transcript can be detected as early as 2 h after 1,25(OH)<sub>2</sub>D<sub>3</sub> addition, consistent with p21<sup>Cip1</sup> being a direct mediator of 1,25(OH)<sub>2</sub>D<sub>3</sub> action. However, in this system, the upregulation of p21<sup>Cip1</sup> after exposure to 1,25(OH)<sub>2</sub>D<sub>3</sub> is relatively transient and accompanied by a proliferative burst [153], which does not correlate with the onset of the G1 block that is observed 24–48 h later and is accompanied by markedly increased levels of p27<sup>Kip1</sup> [152,346,347]. Thus, although

p21<sup>Cip1</sup> may initiate a cascade of unknown events that lead to the expression of the differentiated monocytic phenotype, it is unlikely to be directly responsible for the G1 arrest in leukemia, or SCC cells. Such function, however, has been attributed to p21<sup>Cip1</sup> in other cells, including prostate cancer [361–363], breast cancer [364,365], parathyroid cells [366], gastric cancer [339], or glioblastoma multiforme cells [367]. The finding that p21<sup>Cip1</sup> binds to the proliferating cell nuclear antigen (PCNA), the processive factor for DNA replication, suggests that this complex might play an important role in maintaining the integrity of the genome [368,369].

The first demonstration that upregulation of p27<sup>Kip1</sup> is associated with G1 arrest, which takes place following 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation, was reported by Wang et al. (Table 86.3) [346]. They showed a sustained increase in p27<sup>Kip1</sup> protein abundance that coincided with the appearance of the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced G1 block in HL60 cells and correlated with reduced kinase activity of CDK6 and CDK2 [346,347]. Further, reductions of the levels of p27<sup>Kip1</sup> by several independent approaches reversed the G1 block, but not the differentiated phenotype [342]. Accordingly, these data clearly show that, at least in HL60 cells, p27<sup>Kip1</sup> controls the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced G1 block, but not the differentiated phenotype.

Similar findings have been obtained in several other hematopoietic cell types, although the data cannot always be so clearly interpreted. For instance, the upregulation of p27<sup>Kip1</sup> is often accompanied by an upregulation of p21<sup>Cip1</sup> [364,370,371]. However, even in situations where p21<sup>Cip1</sup> and p27<sup>Kip1</sup> are both upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> or its analogs, increased levels of p27<sup>Kip1</sup> correlate better with the onset of G1 block than the upregulation of p21<sup>Cip1</sup> (e.g., Refs. [152,346,360,372,373]). This, however, is subject to cell context, a striking example being a report that p27<sup>Kip1</sup> is essential for the antiproliferative action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on primary, but not on immortalized, mouse embryonic fibroblasts [374]. The role of CDKs in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced cell cycle arrest is also difficult to assess because, when present at relatively low levels, p21<sup>Cip1</sup> and p27<sup>Kip1</sup> serve to facilitate complex formation of cyclins D with CDKs, and their transport to the nucleus [375,376], and only high levels of CDKs are inhibitory [375,377]. Thus, one possible explanation for the upregulation of p21<sup>Cip1</sup>, which may not correlate with G1 arrest, is that p21<sup>Cip1</sup> simply serves to facilitate cyclin D–CDK complex formation. More likely, however, is that elevated levels of p21<sup>Cip1</sup> inhibit cyclin E–CDK2 activity and therefore block cyclin E–CDK2 phosphorylation of p27<sup>Kip1</sup>, which then leads to degradation of p27<sup>Kip1</sup> in proliferating cells [378–380].

Unlike p21<sup>Cip1</sup>, which can be directly upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> through a VDRE in p21<sup>Cip1</sup> promoter [152], p27<sup>Kip1</sup> has no VDR-binding element in its promoter and may be regulated in a cell type–specific and condition-specific manner by several mechanisms, which include both transcriptional and posttranscriptional levels as discussed before and illustrated in Figs. 86.2 and 86.4. The known transcriptional regulation of p27<sup>Kip1</sup> expression includes at least two mechanisms that can be influenced by 1,25(OH)<sub>2</sub>D<sub>3</sub>, though in opposing ways. One proposal is that transcription factors SP1 and NF-Y can synergistically mediate the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced expression of p27<sup>Kip1</sup> by acting directly on the p27<sup>Kip1</sup> promoter, as shown in

transiently transfected U937 leukemia cells [273]. In these experiments, deletion and mutational analysis revealed that p27<sup>Kip1</sup> promoter activation required both GGGCGG (SP1 binding) and CCAAT (NF-Y binding) sequences. Although p27<sup>Kip1</sup> promoter does not contain VDRE, Huang et al. [225] have shown that transfection of VDR in SW620 colon cancer cells, which express low level of endogenous VDR, enhances 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced p27<sup>Kip1</sup> promoter activation, and that VDR and SP1 cooperate to activate p27<sup>Kip1</sup> expression in LNCaP prostate cancer cells. Particularly, it was demonstrated that VDR physically interacts with SP1 to activate p27<sup>Kip1</sup> promoter via a GC-rich SP1 site [225]. As presented before, SP1 transcription factor is activated in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated leukemia cells; thus, these data could potentially be a plausible mechanism for the induction of G1 arrest by 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced p27<sup>Kip1</sup>, at least in myeloid leukemia cells. The second transcriptional mechanism that can be regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> is based on the demonstration that in a number of cell types, p27<sup>Kip1</sup> is transcriptionally activated by the forkhead transcription factors, such as AFX (FOXO4) or FOXO1 [381,382], and AKT can phosphorylate and thus turn off the FOXO factors [383] allowing cell cycle progress. Interestingly, AKT can be upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [177], and thus provide an opportunity for the cells to mitigate the signals that tend to upregulate p27<sup>Kip1</sup> expression. There appear to be multiple signals for upregulation of p27<sup>Kip1</sup> expression including the AP-1 transcription factor [384], even though the precise site of its transcription origin is uncertain [385]. In addition, translational regulation of p27<sup>Kip1</sup> by the far upstream element-binding protein 1 (FBP1) binding to the unusually long 5'UTR has been reported [385].

However, as illustrated in Figs. 86.2 and 86.4, the dominant form of regulation of p27<sup>Kip1</sup> expression by 1,25(OH)<sub>2</sub>D<sub>3</sub> and other VDDs appears to be posttranscriptional, including miRNA inhibition of translation, and proteasome-dependent protein degradation. For instance, miRNAs-181 were shown to regulate the expression of p27<sup>Kip1</sup> in human myeloid leukemia cells induced to differentiate by 1,25(OH)<sub>2</sub>D<sub>3</sub> [20], as further discussed in the following. Regarding its degradation, in mouse SCC AT-84 cells, the analog EB1089 did not change p27<sup>Kip1</sup> mRNA levels, but reduced the miRNAs for p45<sup>Skp2</sup>, which ubiquitinates p27<sup>Kip1</sup>, and for CKS1, which targets p45<sup>Skp2</sup> to p27<sup>Kip1</sup> [386,387]. A similar decrease in p45<sup>Skp2</sup> expression and stabilization of p27<sup>Kip1</sup> protein was demonstrated in acute promyelocytic leukemia cells [387], ovarian cancer cells [388], and human hepatoma cells [389]. Since these changes become evident at about 48 h of the exposure to the analog, there is good correlation with the onset of the G1 block. The latent period of 24–48 h for p27<sup>Kip1</sup>



upregulation may also be needed to inactivate the cyclin E-CDK2 complex, which phosphorylates Thr187 of p27<sup>Kip1</sup> that under some conditions is required for ubiquitination of p27<sup>Kip2</sup> by p45<sup>Skp2</sup> [378]. Inhibition of cyclin E-CDK2 activity following exposure of HL60 cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> has been demonstrated [347], and this may contribute to 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced increase in p27<sup>Kip1</sup> levels. However, p27<sup>Kip1</sup> can be phosphorylated on non-CDK sites [390–392], so this form of control may have redundancy.

Another form of regulation of p27<sup>Kip1</sup> was first discovered in myeloid cells. Wang et al. found that the members of the miRNA-181 family can inhibit the expression of p27<sup>Kip1</sup>, but exposure of the cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces the levels of these miRNAs and thus leads to an increase in the levels of p27<sup>Kip1</sup> and cell cycle arrest [20] (Figs. 86.2 and 86.4). As mentioned before, this role of miRNA-181a was confirmed by Cuesta et al. in myeloid cells that were treated with TPA and subsequently induced to differentiate into the macrophage differentiation phenotype [393]. Further, regulation of p27<sup>Kip1</sup> by several miRNAs was shown in other leukemic cells [394–396]. It seems that such multiple controls by 1,25(OH)<sub>2</sub>D<sub>3</sub> of p27<sup>Kip1</sup> expression signify an especially critical role of 1,25(OH)<sub>2</sub>D<sub>3</sub> in cell cycle regulation.

#### **4.3.3 Retinoblastoma protein control of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced G1 block**

The suggested placement of the inactivation of the cyclin E-CDK2 complex upstream of p27<sup>Kip1</sup> upregulation raises the question of how this complex is inactivated in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated cells. One possible answer is provided by the finding that the RB gene is upregulated early in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation of HL60 cells [37], as illustrated in Fig. 86.4. The increased levels of pRB can then bind and inactivate E2F transcription factors necessary for the expression of cyclin E, and thus the activity of the cyclin E-CDK2 complex (Fig. 86.4). Accordingly, the phosphorylation of Thr187 on p27<sup>Kip1</sup> is reduced, allowing the accumulation of this CDKI, and a further increase in the hypophosphorylated forms of pRB, also a substrate for the cyclin E-CDK complex. Hypophosphorylated pRB now further binds E2F and thus reduces cyclin E expression to the point that p27<sup>Kip1</sup> is no longer phosphorylated and degraded, as the result of this positive feedback loop, leading to G1 arrest. It is known that in HL60 cells, the expression of pRB normally occurs primarily during G1 phase and can be detected at both mRNA and protein levels within 12 h of exposure to 1,25(OH)<sub>2</sub>D<sub>3</sub> [37,293] although the mechanism of its upregulation remains to be determined. These in vitro studies are supported by the finding of gross defects in the development of the hematopoietic system in RB-

KO mice [406,407], and by the transcriptional studies that show that the RB gene plays a role in normal human adult hematopoiesis [408]. Thus, pRB appears to have a role in the early stage of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation, and to contribute to changes in cellular transcriptional and kinase activities that lead to G1 arrest at a later stage [409].

#### **4.3.4 Downregulation of c-MYC expression in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation and G1 arrest**

The pRB/E2F pathway also controls the expression of c-MYC, as E2F transcription factors upregulate the c-MYC gene, and inhibition of c-MYC expression by 1,25(OH)<sub>2</sub>D<sub>3</sub> may be responsible, at least in part, for the G1 block in differentiating cells (Fig. 86.4) [410]. Indeed, the association between c-MYC downregulation and 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation of human leukemia cells was one of the earliest findings that initiated the studies of the molecular basis of the cellular changes that follow exposure to this hormone [2,280,411,412]. Subsequently, several studies have shown a marked reduction in c-MYC expression in response to 1,25(OH)<sub>2</sub>D<sub>3</sub> and analog treatment of colon cancer cells [413,414] and prostate cancer cells [348,415], and this was associated with the induction of differentiation [414] and G1 arrest [415]. The intense interest in c-MYC as a potential negative regulator of differentiation was fueled largely by its deregulated expression in several types of human neoplasia [416,417]. c-MYC is known to promote cell cycle progression mostly through coordinated transcriptional regulation of target genes (e.g., Ref. [418]). These include the DNA replication and cell cycle traverse-promoting genes such as ornithine decarboxylase, CDC25A, and cyclins E and A [416]. Conversely, c-MYC inhibits the transcription of cell cycle inhibitor p21Cip1 [419], and it has been suggested that this is due, at least in part, by sequestering the SP1 transcription factor [420] or by inhibiting ERK and P70S6K/4EBP1 phosphorylation [421], which are required for p21Cip1 transcription. While the aforementioned considerations present an almost complete sequence of events that can explain the G1 arrest induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> (summarized in Fig. 86.4), an additional level of control of c-MYC expression by 1,25(OH)<sub>2</sub>D<sub>3</sub> is provided by studies of Simpson et al. [422]. They found that in differentiating HL60 cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> increased the expression and DNA-binding activity of HOXB4, a product of a homeobox gene, and that HOXB4 binds to the sites in the c-MYC gene, which are involved in blocking by 1,25(OH)<sub>2</sub>D<sub>3</sub> of the elongation of c-MYC transcripts [284]. Further, these authors demonstrated that a HOXB4 antisense oligonucleotide partially inhibited the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced decrease in c-MYC protein levels [423]. While

they observed reduction of  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation in these experiments, the effect of HOXB4 antisense on the G1 block was not reported. Nonetheless, these studies are significant, as members of the HOX gene family are known to be involved in hematopoiesis and leukemogenesis [424–426]. Also, other HOX genes participate in  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation; HOXB7 was reported to increase in HL60 cells [427], while in U937 and MCF-7 cells  $1,25(\text{OH})_2\text{D}_3$  increased expression of HOXA10 [285], and the Iroquois homeobox gene 5 (IRX5) is regulated by  $1,25(\text{OH})_2\text{D}_3$  in LNCaP human prostate cancer cells, and controls the cell cycle as well as apoptosis [428].

Available data suggest that  $1,25(\text{OH})_2\text{D}_3$  may inhibit colon cancer cell proliferation by inhibiting the APC/ $\beta$ -catenin pathway. For instance, in SW480 cells,  $1,25(\text{OH})_2\text{D}_3$  promotes VDR/ $\beta$ -catenin interaction and prevents  $\beta$ -catenin nuclear translocation, leading to inhibition of TCF-4 responsive genes such as c-MYC [55], a proto-oncogene required for formation of tumors in many settings. Thus, transcriptional blockage of c-MYC expression in these cells after exposure to  $1,25(\text{OH})_2\text{D}_3$  [413] may be associated with modulation of the APC/ $\beta$ -catenin signaling pathway. Another mechanism by which  $1,25(\text{OH})_2\text{D}_3$  can downregulate c-MYC expression was suggested by Rohan et al. [348], who demonstrated that treatment of C4-2 prostate cancer cells with  $1,25(\text{OH})_2\text{D}_3$  resulted in a 50% decrease in c-MYC mRNA but a much more extensive reduction in c-MYC protein. Further experiments showed that decreased c-MYC stability was due to an increase in the proportion of c-MYC phosphorylated on Thr58, a glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) site that serves as a signal for ubiquitin-mediated proteolysis. Thus, it appears that  $1,25(\text{OH})_2\text{D}_3$  regulates c-MYC levels in cancer cells by several different pathways, which involve both a reduction in c-MYC gene expression and a decrease in c-MYC protein stability.

#### 4.3.5 G2/M retardation and polyploidization

The occurrence of abnormalities in G2/M transition in  $1,25(\text{OH})_2\text{D}_3$ -treated cells has been observed infrequently, with a general consensus that the G1 phase is the principal target of the antiproliferative actions of  $1,25(\text{OH})_2\text{D}_3$  and its analogs. However, in early studies of  $1,25(\text{OH})_2\text{D}_3$  action, Abe et al. [187] detected an increase in the G2/M compartment in WEHI murine myelomonocytic cells, also described in HL60 cells by Godyn et al. [306]. The basis for this increase may be a reduction in the levels of CDK1 in these cells [429], although the roles of cohesin, separase, or PLKs remain to be investigated in the light of the recently accumulating knowledge of mitotic controls. The  $1,25(\text{OH})_2\text{D}_3$ -induced G2 arrest is also seen in LNCaP prostate cancer cells, which is due to  $1,25(\text{OH})_2\text{D}_3$ -mediated upregulation of

miRNA-98, a miRNA with tumor suppressor properties [430]. The combination of  $1,25(\text{OH})_2\text{D}_3$  and PS121912, a VDR coactivator inhibitor, arrested HL60 cells in S and G2 by downregulating E2F1 and E2F4, causing the downregulation of cyclins D and A and subsequent cell cycle arrest [431].

One consequence is the higher ploidy of HL60 cells exposed for prolonged periods of time to  $1,25(\text{OH})_2\text{D}_3$ , observed as an increased number of binucleated cells [306], or as nearly doubled DNA content of these cells [254]. Interestingly, polyploidization of  $1,25(\text{OH})_2\text{D}_3$ -treated cells is an alternative to differentiation, as these cells override the antiproliferative actions of  $1,25(\text{OH})_2\text{D}_3$  and do not express differentiated phenotype [254]. Thus, HL60 cells can have a  $1,25(\text{OH})_2\text{D}_3$ -induced defect in completion of mitosis that allows one round of DNA endoreduplication. Whether osteoclast, or perhaps megakaryocyte, polyploidization is also influenced by  $1,25(\text{OH})_2\text{D}_3$  remains a possibility.

#### 4.4 Inhibition of cell proliferation by vitamin D and analogs without evidence of differentiation: cell type specificity

While some effects of  $1,25(\text{OH})_2\text{D}_3$  and its analogs can be recognized in a variety of cell types, there is remarkable cell type specificity of most of such effects, and it is important to realize that only a few generalizations can be made regarding the antiproliferative actions of these compounds. However, it appears to be true that  $1,25(\text{OH})_2\text{D}_3$ -induced differentiation is not simply a consequence of inhibited proliferation, as differentiation often precedes the G1 block [307], and  $1,25(\text{OH})_2\text{D}_3$  can inhibit cell proliferation with only minimal, or absent, evidence of differentiation. Indeed, the antiproliferative effect of  $1,25(\text{OH})_2\text{D}_3$  on cultured melanoma cells was recognized by Colston et al. [432] in 1981, at the same time as the differentiation-inducing action of  $1,25(\text{OH})_2\text{D}_3$  was described in myeloid leukemia cells by Abe et al. [1]. A recent example of inhibition of growth without differentiation is the report that in human airway, smooth muscle cells  $1,25(\text{OH})_2\text{D}_3$  decreased PDGF-induced cell growth by inhibiting pRB and CHK1 phosphorylation [433]. Cell specificity of responses to  $1,25(\text{OH})_2\text{D}_3$  is also illustrated by the finding that  $1,25(\text{OH})_2\text{D}_3$  can cause a G1 block in cultured thyroid carcinoma and pituitary corticotroph, but not lactotroph, cells [434,435]. Interestingly, while in thyroid carcinoma cells, the mechanisms of the antiproliferative effects include dephosphorylation of p27<sup>Kip1</sup> in a PTEN-dependent manner, leading to a diminished association between p45<sup>Skp2</sup> and p27<sup>Kip1</sup> with its consequent accumulation [434], in pituitary corticotroph cells, the mechanism appears to be a diminished association of

p27<sup>Kip1</sup> with p45<sup>Skp2</sup> and CDK2, without an involvement of PTEN [435]. This illustrates not only the exquisite cell type specificity of the mechanisms involved in the antiproliferative actions of vitamin D and its analogs, but also that the upregulation of p21<sup>Kip1</sup> is unrelated to differentiation, as demonstrated previously in leukemia cells [342].

Another mechanism for the antiproliferative actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> on cell types that show only minimal evidence of differentiation is provided by the apoptosis-inducing actions of vitamin D and its analogs, as described in the next section and in other chapters in this volume. Again, cell type determines this response, as in contrast to various carcinomas, e.g., breast cancer cells [436], 1,25(OH)<sub>2</sub>D<sub>3</sub> protects HL60 leukemia cells from apoptosis, as first demonstrated by Xu et al. [437] and further confirmed by subsequent studies in both HL60 cells [177,178,438,439] and other cancer [440] and noncancer [441–444] cell types. Thus, the activity of survival pathways may determine whether differentiation can take place in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>, or whether a potential default pathway will lead to apoptosis, perhaps as the result of prolonged residence in a cell cycle compartment other than G0. In any case, 1,25(OH)<sub>2</sub>D<sub>3</sub> can be an effective antiproliferative agent in many cell types that express VDR.

## 5. Effects of VDDs on cell survival and cell death

### 5.1 VDDs can increase cell death

Depending on the cell type, VDDs can either protect the cells from death or lead to their demise. For instance, VDDs have been reported to induce apoptosis in cells derived from murine squamous cell carcinoma [445], in cell lines derived from human breast cancer cells [446], or human hepatocellular carcinoma (HCC) cells [447]. VDDs can also activate genes and proteins responsible for apoptosis of other cell types, such as colon and prostate cancer cells [448,449]. Many reports presented evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs activate the expression of proapoptotic proteins BAK and BAX and suppress the antiapoptotic BCL family members, BCL2 and BCL-xL [450,451]. This causes the efflux of cytochrome c from the mitochondria to the cytoplasm and triggers the activation of the downstream executor caspases such as caspase-3, as well as the upstream initiator protease caspase-9, and induction of apoptosis [452]. The mechanism of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced apoptosis varies with the cell type and can be mediated by either p53-dependent, or independent pathways [453–456]. Moreover, it has been shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> may induce apoptosis by dysregulation of the signaling pathways activated by different growth factors and its

receptors. For example, it downregulates insulin-like growth factor receptor (IGFR) [457] as well as upregulates tumor necrosis factor alpha (TNFα) [458].

It was also shown that either 1,25(OH)<sub>2</sub>D<sub>3</sub> or its analogs, EB1089 and ILX23-7553, can potentiate the response to ionizing irradiation [456,459–461]. For the analog EB1089, such activity was detected in MCF-7 breast tumor xenografts in nude mice [462]. In these studies, the effect of EB1089 combined with radiation on growth of human breast cancer xenografts was greater than radiation alone, raising the possibility that EB1089 interfered with DNA repair. It seems that cell death induced by irradiation in the presence of EB1089 is a consequence of alterations in signaling pathways downstream of the DNA damage. These signaling pathways may also involve the generation of reactive oxygen species, acceleration of cell senescence, and c-MYC independent apoptosis, as well as p53-dependent cell death by autophagy [461].

### 5.2 VDDs can increase cell survival

In contrast to studies described before, AML cells are protected from death by VDDs [439]. Other examples include fibroblasts, keratinocytes, and primary melanocytes, in which 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation is accompanied by increased cell survival and decreased apoptosis [177,178,439,463]. In melanocytes, sphingosine 1-phosphate was identified as a downstream mediator of 1,25(OH)<sub>2</sub>D<sub>3</sub> actions [463]. Interestingly, in mice, 1,25(OH)<sub>2</sub>D<sub>3</sub> administration prevented bleomycin-inducing lung interstitial tissue damage that leads to lung fibrosis [464].

The older literature also showed that 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation of AML cells can be divided into several phases. During the initial phase, the cells proliferate normally, and cell cycle progression is driven by the high levels of MEK1/2, ERK1/2, and JNKs [183,184,251]. At the later phase, c-RAF protein activates ribosomal S6 kinase p90RSK that together with other kinases, such as ERK1/2 and ERK5, activates the master transcription factor for monocytes/macrophages differentiation, C/EBPβ [33,192,294]. Prosurvival signals transmitted from RAF1 to downstream targets are augmented by KSR1 and KSR2, essential for RAF1 activation and/or phosphorylation [154,155,465–467]. KSR2 knockdown decreases cell survival, which is accompanied by reduced BCL2/BAX and BCL2/BAD ratios and increased cleavage of caspase-3 [439]. Moreover, during the later phase of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation of AML cells, elevated expression of other antiapoptotic proteins, BCL-xL and MCL1, facilitates differentiation by increasing cell survival [178]. Additionally, in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiating AML cells,



expression of proapoptotic protein BIM is inhibited by miRNA-32 [468].

In the more recent literature, it seems to be a general finding that in cells with DNA damage, exposure to VDDs enhances cell death [469,470]. This was examined in detail in AML cells treated by AraC, the standard chemotherapeutic agent for this disease, and remarkably, the enhancement of cell death was selective for malignant blasts, as the AraC-induced cell death of normal bone marrow cells was not increased by VDDs [471,472]. In these experiments, the effect was potentiated by combination of a vitamin D<sub>2</sub> derivative, doxercalciferol (Dox) with a botanical, carnosic acid (CA) [471,472]. It is likely that the survival enhancing action of the combination (Dox/CA) is primarily due to the increased expression of VDR in the presence of CA, as a previous publication demonstrated that CA markedly increases the levels of VDR protein [473]. The requirement for VDR to obtain an optimal cell death-enhancing effect was also shown by siVDR transfection of AML cells [472]. The initial studies of the molecular mechanisms demonstrated that the enhancement of cell death by the differentiation-inducing combination Dox/CA was executed largely by caspase-3, activated downstream from the proapoptotic SH3-only protein BIM, most likely via caspase-9 [472]. It was speculated that the choice between DNA repair and apoptosis can be determined by the Tyr-142 phosphorylation of the DNA damage-related protein H2AX [474]. In this scenario, the DNA repair–apoptosis switch depends on several factors, including the binding of JNK stress-related protein kinase to H2AX phosphorylated on Tyr-142. This then leads to apoptosis in the system studied. The role of VDDs in this experimental system remains to be investigated, but since VDDs upregulated JNK expression in AML cells [259,475,476]. Recent studies in AML cells revealed that direct activation of ASK1 kinase by thioredoxin-interacting protein (TXNIP) is required for the optimal transmission of the cell death signal to apoptotic machinery and is regulated by JNK and BIM [477]. This is an interesting possibility for VDDs-based clinical applications.

### 5.3 Autophagy and VDDs

In addition to its effects on apoptosis, 1,25(OH)<sub>2</sub>D<sub>3</sub> can regulate the induction of autophagy, which affects cell survival, although the details vary significantly depending on the type of cell and cellular stress. In some circumstances, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the cytoprotective effects of autophagy and facilitates cell death, which can be advantageous in the design of cancer chemotherapy regimens. In breast and non–small-cell lung cancer cell lines exposed to radiation, both

1,25(OH)<sub>2</sub>D<sub>3</sub> and its analog EB1089 promote a prolonged cell cycle arrest, inhibit the normal proliferation-related recovery from radiation damage, and reduce the normal cytoprotective effects of autophagy, thus enhancing the effectiveness of radiation treatment against these tumor cells [478].

However, in most cells studied, 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates autophagy and promotes cell survival. In cardiomyocytes, 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated VDR activation protected against ischemia/reperfusion injury by promoting autophagy, mediated in part by supporting the enhanced activation of Beclin-1 [479]. In human monocytes and macrophages, 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates expression of the ASAP2 gene, which encodes a multi-domain protein that localizes in the Golgi and plasma membrane and is involved in vesicle transport and autophagy [480]. In endothelial cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> may prevent cell death by reducing the expression of apoptosis-inducing proteins and upregulating the expression of Beclin-1, thus enhancing autophagy and downregulating apoptosis [481]. Most relevant to this chapter, in macrophages, 1,25(OH)<sub>2</sub>D<sub>3</sub> and a functional VDR are critical in activating autophagy and autophagic defenses against mycobacteria infection [482]. Normal macrophages demonstrate significant release of TNF $\alpha$ , I- $\kappa$ B degradation, and NF- $\kappa$ B nuclear translocation in response to *Mycobacterium tuberculosis* infection. This response is greatly reduced in HIV-infected macrophages, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> pretreatment restores TNF $\alpha$  release and NF- $\kappa$ B nuclear translocation [483]. 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated autophagy may also play a role in slowing HIV infection. For instance, in macrophages, TLR8 ligands activate a 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated autophagic response that inhibits HIV-1 infection [484]. Also, it has been reported that an intraperitoneal injection of 25(OH)D<sub>3</sub> protects mouse skin from UV damage. This effect is mediated chiefly by antiinflammatory M2 macrophages [485].

In another system, 1,25(OH)<sub>2</sub>D<sub>3</sub> was found to trigger an “autophagic switch” and reprogram ZR-75-1 breast cancer cells from radiation alone–induced cytoprotective autophagy to cytotoxic autophagy when treated with radiation plus 1,25(OH)<sub>2</sub>D<sub>3</sub> [486,487]. This interesting concept may encourage similar studies in hematopoietic cells, e.g., in reference to health disparities, as autophagy regulation by 1,25(OH)<sub>2</sub>D<sub>3</sub> in primary monocytes–macrophages appears to be varying between black and white individuals [488].

## 6. Effects of VDDs on lymphoid lineage cells and immunity

While the bulk of studies of the effects of VDDs and their therapeutic potential in hematologic malignancies



focus on the myeloid lineage hematopoietic cells, lymphoid cells can also recognize the presence of VDDs and respond in various ways.

### 6.1 Immunoregulatory actions of VDDs on hematological malignancies: reversing the leukemia-induced immunodeficiency

All immune cells are progeny of the hematopoietic stem cells (HSCs), which, when driven by intrinsic and extrinsic signals in the bone marrow, undergo differentiation steps to produce various types of blood and immune cells [489].

The character of the immune dysfunction in hematological malignancies depends on the type of the disease. For instance, in myeloid leukemias, the dysfunction affects innate, while in lymphoid leukemias an acquired immune system, due to the replacement of immune cells by cancer cells. Thus, all leukemias, and especially acute types, are accompanied by an increased risk of infections [490].

The primary goal in the treatment of leukemias is an elimination of rapidly proliferating leukemic blasts. However, chemo- or radiotherapy not only removes proliferating blasts but also prevents the remaining immune cells from being activated, since immune activation is always accompanied by a proliferation. One of the most common, and most dangerous consequences, of chemotherapy is neutropenia, which results in severe fungal infections [491]. In this way, chemotherapy-induced immunodeficiency adds to the leukemia-induced immunodeficiency.

The need to improve functions of the immune system has been appreciated predominantly in patients with chronic lymphocytic leukemia, the disease in which lenalidomide appeared to have two activities, antitumor and immunostimulatory [492]. The idea to apply other compounds, which stimulate development and function of immune cells, is rational. One class of such compounds is  $1,25(\text{OH})_2\text{D}_3$  and low-calcemic VDDs.

#### 6.1.1 $1,25(\text{OH})_2\text{D}_3$ and hematopoiesis

The presence of  $1,25(\text{OH})_2\text{D}_3$  seems not to be essential for human hematopoiesis, since as mentioned before, VDR-KO mice have normal numbers of all types of blood cells, but these mice are not able to respond properly to pathogens [12]. When exposed to pathogens, the mice without VDR respond with production of chronic myeloid leukemia-like cells, splenomegaly, granulocytosis, thrombocytosis, and reduced erythropoiesis [493]. However, both murine and human HSCs express VDR, and the receptor is transcriptionally active in these cells [291]. The physiological level of  $1,25(\text{OH})_2\text{D}_3$  is

necessary to keep HSCs in a proliferating state [15] and to induce monocytic markers of differentiation [13]. The aforementioned facts prompted investigation of supporting hematopoiesis after autologous stem cell transplantation (HSCT) using  $1,25(\text{OH})_2\text{D}_3$ . The results of the randomized study performed on 80 patients after HSCT revealed that  $1,25(\text{OH})_2\text{D}_3$  improved lymphocytes recovery and a 2-year relapse-free survival in patients, without producing adverse side effects [494]. Not only did  $1,25(\text{OH})_2\text{D}_3$  improve patient recovery after HSCT, but it also reduced a risk of graft versus host disease (GVHD) [495,496].

#### 6.1.2 $1,25(\text{OH})_2\text{D}_3$ and innate immunity

The capability of  $1,25(\text{OH})_2\text{D}_3$  to regulate human immune system has been known for many years. However, the ability to perform genome-wide analyses have provided deeper insight to understanding this process [497]. These studies performed in cancer cell lines have revealed that among hundreds of VDR-regulated genes, many belong to the immune system [47,498]. Many of these immune-related genes were upregulated by  $1,25(\text{OH})_2\text{D}_3$  in blood cells of healthy people, as well [47,499]. The cells that appear to be the most important targets of  $1,25(\text{OH})_2\text{D}_3$  in the immune system are macrophages and dendritic cells. These cells not only have high expression of VDR [32] but are also able to produce  $1,25(\text{OH})_2\text{D}_3$  from its precursor  $25(\text{OH})\text{D}_3$  [500]. Furthermore,  $1,25(\text{OH})_2\text{D}_3$  is capable of directly regulating the expression of genes responsible for monocyte/macrophage functions, such as CD14 [501,502], cathelicidin [503], or  $\text{TNF}\alpha$  [504]. This ability supports the immune defense against *M. tuberculosis* [503], dengue virus [505], HIV [506], or cancer [507]. However, the influence of  $1,25(\text{OH})_2\text{D}_3$  toward human innate immune response is more complex than just the upregulation of the immune-related genes. It has been shown that pretreatment of human monocytes with  $1,25(\text{OH})_2\text{D}_3$  reduces their production of IL-6 and  $\text{TNF}\alpha$  in response to bacterial lipopolysaccharide (LPS) [508]. There are also reports that suggest that the physiological levels of vitamin D may prevent damage in the human body caused by a “cytokine storm” in response to viral infections [509]. Indeed, several groups have reported that the incidence and the severity of COVID-19 infections correlate with vitamin D insufficiency [510–514].

#### 6.1.3 $1,25(\text{OH})_2\text{D}_3$ and acquired immunity

As indicated before, murine T and B lymphocytes express the VDR gene, however, at a lower level than HSCs and thymocytes [515]. Nevertheless, many studies have revealed that in mice and in humans, all cell types of the acquired immune system respond to  $1,25(\text{OH})_2\text{D}_3$ .

### 6.1.3.1 T lymphocytes

Subsets of T lymphocytes (T cells), including both resting T cells (expressing either CD8 or CD4) and activated T cells, express VDR [129,516,517]. VDR mRNA expression increases when these cells are stimulated to proliferate, for example, after their exposure to the mitogen phytohemagglutinin-A (PHA) for 24 h *in vitro*. VDR mRNA was detected by RT-PCR in murine intraepithelial lymphocytes and regulatory T cells (Treg) expressing FoxP3, CD4, and CD25 [517,518].

Several studies of VDR-KO mice provided insights into the role of VDR in lymphoid cells [12,519]. Although the proportions of T and B cells are normal in the VDR-KO mice, antigen-stimulated spleen cells from VDR-KO mice produce less interferon ( $\text{IFN}\gamma$ ) and more IL-4 than those from WT mice, indicating impaired Th1 differentiation. Additionally, IL-12 stimulation induces a weaker proliferative response in VDR-KO splenocytes than those in WT mice, and expression of STAT4 is reduced. These results suggested that VDR plays an important role in the Th1-type immune response, but not in thymocyte development.

Tregs are phenotypically, functionally, and quantitatively normal in VDR-KO mice. However, the number of intraepithelial lymphocytes is reduced in VDR-KO mice, resulting in the failure of T cell homing into the gastrointestinal tract, and this possibly increases a risk of developing inflammatory bowel disease [518]. Tregs, an important cell subset of T cells, respond to  $1,25(\text{OH})_2\text{D}_3$  by a shift in their polarization, increasing percentages of Th2 and Treg cells, and decreasing percentages of Th1 and Th17 cells in peripheral blood of rodents [520,521]. This mechanism is most likely responsible for the beneficial effects of  $1,25(\text{OH})_2\text{D}_3$  that have been observed in patients with Th1-mediated autoimmune diseases, such as type 1 diabetes mellitus [522], multiple sclerosis [523], or psoriasis [524]. The precise mechanism of T cell polarization in response to  $1,25(\text{OH})_2\text{D}_3$  has not been fully elucidated, but  $1,25(\text{OH})_2\text{D}_3$ -induced increases in the expression of FOXP3, IL-10, and TGF- $\beta$ 1 in T cells were observed [525].

A report on VDR-KO mice showed that pathogen-specific effector and memory CD8 T cells respond differently to intracellular viral and bacterial pathogens in the presence or absence of  $1,25(\text{OH})_2\text{D}_3$  signals. However, the  $1,25(\text{OH})_2\text{D}_3$  signals serve to regulate antigen-specific effector and memory CD8 T cell size, repertoire, and survival. One mechanism by which  $1,25(\text{OH})_2\text{D}_3$  mediates the T cell repertoire is the inhibition of CYP11A1 transcription by  $1,25(\text{OH})_2\text{D}_3$ -bound VDR via interactions with the CYP11A1 promoter [526]. CYP11A1 facilitates the IL-4-mediated conversion of  $\text{IFN-}\gamma$  secreting CD8+ T cells (Tc1) to IL-13 secreting CD8+ T cells (Tc2); the former more involved in

antitumor activity, and the latter more involved in allergic and asthmatic responses. The use of VDR-KO mice also showed that there are potential regulatory connections between VDDs and effector as well as memory CD8 T cell differentiation events during infections [527]. The absence of  $1,25(\text{OH})_2\text{D}_3$  signals leads to aberrant CD8 T cell effector differentiation, enhanced contraction in antigen-specific cytotoxic T lymphocytes (CTLs), a significantly restricted breadth of the antigen-specific CD8 T cell effector and memory repertoire, and preferential localization of effector and memory CD8 T cells to the lymph nodes compared with nonlymphoid tissues [519,527]. The implications of the aforementioned findings for malignant disease may relate to the functional state of host defenses against aberrant cells due to their neoplastic transformation.

### 6.1.3.2 B lymphocytes

B lymphocytes are also regulated in response to  $1,25(\text{OH})_2\text{D}_3$ . In humans,  $1,25(\text{OH})_2\text{D}_3$  has been shown to inhibit proliferation and enhance apoptosis of activated B cells and to inhibit production of immunoglobulins (Ig) by plasma cells [528]. The influence of  $1,25(\text{OH})_2\text{D}_3$  is most probably mediated by IL-10, the cytokine that inhibits B cells migration, induces class switch from IgE to IgM [529]. IgG and IgA also block activation of cytotoxic T cells [529].

The regulation of VDR expression in B cells is principally at the transcriptional level [129,516]. No VDR mRNA or protein is detected in resting B lymphocytes, for example, in normal human B cells from tonsils, until their activation [516,530]. *In vitro*  $1,25(\text{OH})_2\text{D}_3$  inhibits the synthesis of Ig by plasma cells [402,528]. Their inhibition may be mediated through the activation of VDR/RXR in these cells, and/or through the inhibition of Th helper cell activity [531]. Production of lymphokines, including IL-2, is markedly decreased by  $1,25(\text{OH})_2\text{D}_3$  in activated T lymphocytes, and this could cause the suppression of Ig synthesis [532–534].

Non-Hodgkin's lymphoma and Burkitt's lymphoma B cell lines have been shown to express only low levels of VDR; yet  $1,25(\text{OH})_2\text{D}_3$  ( $10^{-7}$  M) can decrease the proliferation of these cells *in vitro* [535]. More recently, it has been observed that low levels ( $<20$  ng/mL) of serum  $25(\text{OH})\text{D}_3$  are associated with reduced survival of patients with follicular lymphoma [536]. Clearly, further investigations are needed to determine the effects of vitamin D supplementation in this clinical setting.

The immunomodulatory actions of  $1,25(\text{OH})_2\text{D}_3$  can influence not only diseases of the immune system but also neoplasms. The devastating role of cancer-associated inflammation, which creates an indispensable and important environment for the growth of

neoplasms, has been very well documented [537]. Cancer cells secrete various cytokines and growth factors, which attract immune cells to the tumor site, support its development, and create an environment where angiogenesis and lymphangiogenesis are promoted [538]. Inhibition of the devastating inflammation by  $1,25(\text{OH})_2\text{D}_3$  and its low-calcemic analogs, accompanied by an enhanced degradation of pathogens, suggests that these compounds can be promising agents to support anticancer treatment.

## 7. Clinical applications of VDD actions against hematopoietic malignancies

### 7.1 Differentiation therapy of leukemia

A typical abnormality of leukemic cells is that they harbor a block at an early stage of HSC development and fail to differentiate into functional mature cells. As discussed before, numerous early studies popularized the strategy of inducing differentiation and growth arrest as an alternative to inducing the death of cancer cells by cytotoxic therapies. The potential for differentiation therapy to improve outcomes in leukemia is exemplified by the development of therapy with ATRA for the targeted treatment of APL, also known as AML-M3. ATRA is specifically effective in APL cells carrying a typical chromosomal translocation between chromosomes 15 and 17 (t [15,17](q22;q21)), the fusion product of which is PML-RAR $\alpha$ . The leukemia with translocation NPM/RAR $\alpha$ , but not other leukemias including PLZF/RAR $\alpha$  translocation leukemia, is also responsive to treatments with ATRA, ATRA/As $_2$ O $_3$ , or ATRA together with other compounds [539].

Since  $1,25(\text{OH})_2\text{D}_3$  was first noted to induce leukemia cell differentiation [1] and extends the survival of mice inoculated with these cells [540], numerous findings obtained in various models of cancer demonstrated that  $1,25(\text{OH})_2\text{D}_3$  can be an effective anticancer agent [4,541,542]. However, the mechanistic basis for clinical trials of VDDs as potential drugs for differentiation therapy of leukemia and other malignancies is still being developed.

### 7.2 VDDs and their functional analysis

A problem with using  $1,25(\text{OH})_2\text{D}_3$  is its hypercalcemic effect, which prevents clinical use of doses effective in arresting or killing cancer cells in model systems. To overcome this problem, many analogs of  $1,25(\text{OH})_2\text{D}_3$  have been synthesized that have enhanced potency to inhibit proliferation and promote differentiation of cancer cells but have lower calcemic activity than the parent compound [541,542]. Vitamin D $_2$  (ergocalciferol) has

been shown to exhibit essentially the same general biological effects as vitamin D $_3$  (cholecalciferol), though there is some controversy regarding relative biopotencies of the two compounds in model systems and in humans [543,544]; also reviewed in Ref. [545]. A direct comparison of the two  $1,25$ -dihydroxyvitamin D derivatives,  $1,25(\text{OH})_2\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_2$ , at spectrophotometrically validated concentrations in a panel of AML cell lines representing different stages of myeloid maturation (KG-1a, HL60, U937, MOLM-13) revealed significantly lower prodifferentiation potency of  $1,25(\text{OH})_2\text{D}_2$  [546]. On the other hand, there is evidence that vitamin D $_2$  and its derivatives are less toxic than vitamin D $_3$  compounds in animals [545,547].

Studies in cell culture in vitro showed that many VDDs are between 10- and 1000-fold more active than  $1,25(\text{OH})_2\text{D}_3$  in their growth-suppressive and differentiation-inducing activity [548,549]. These VDDs can provide a larger therapeutic window for the treatment of hematologic malignancies and should be considered for the selected trials in hematologic malignancies alone, though it is far more likely to be effective in combination with other therapies.

However, it should be remembered that there are important cell type and species differences in the activities of these compounds, and many analogs are no longer available from the manufacturers. Those previously explored and some currently of promise for treatment of AML and myelodysplastic syndrome (MDS) include alfacalcidol, inecalcitol, paricalcitol, doxercalciferol, and others.

#### 7.2.1 Alfacalcidol

The first attempts to use analogs focused on  $1\alpha(\text{OH})\text{D}_3$  (alfacalcidol), a vitamin D $_3$  compound that is efficiently converted to  $1,25(\text{OH})_2\text{D}_3$  in vivo by D $_3$ -25-hydroxylase. Alfacalcidol has been widely used since 1981 as a prodrug for  $1,25(\text{OH})_2\text{D}_3$  in the treatment of hypocalcemia, chronic renal failure, hypoparathyroidism, and osteoporosis [541,550]. This compound effectively induced differentiation and inhibited proliferation of U937 monoblastic leukemia cells [551]. Alfacalcidol was administered to mice inoculated with M1 leukemia cells, and it showed greater antileukemic activity than  $1,25(\text{OH})_2\text{D}_3$  [540]. Its conversion to the active form resulted in a prolonged elevation of plasma levels of  $1,25(\text{OH})_2\text{D}_3$ , and the dosage of 25 pmol every other day, prolonged survival by 50%–60% while producing only a slight increase in the serum calcium.

#### 7.2.2 Inecalcitol

The 19-nor-14-epi-23-yne- $1,25(\text{OH})_2\text{D}_3$  analog of vitamin D $_3$  (inecalcitol) has been first described in 2000 [552] and analyzed in detail in breast cancer [552], prostate cancer [553], and SCC [554] models. In HL60 cell



cultures, inecalcitol was about 6- and 11-fold more potent than  $1,25(\text{OH})_2\text{D}_3$  in inducing cell differentiation [555] and inhibiting proliferation [553], respectively. The maximum tolerated dose was 30  $\mu\text{g}/\text{mouse}$  (i.p., every other day) compared with 0.0625  $\mu\text{g}/\text{mouse}$   $1,25(\text{OH})_2\text{D}_3$ , indicating that inecalcitol was 480 times less calcemic than  $1,25(\text{OH})_2\text{D}_3$  [553]. Similar results were obtained following daily intraperitoneal injections for 7 days [555]. Inecalcitol treatment of mice inoculated with breast [552], SCC [554], and prostate [553] cancer cells demonstrated a marked reduction in tumor growth with minimal calcemic effects.

### 7.2.3 Paricalcitol

Paricalcitol (19-nor- $1,25(\text{OH})_2\text{D}_2$ ) has been approved by the US Food and Drug Administration for the clinical treatment of secondary hyperparathyroidism. Studies by several groups have demonstrated that paricalcitol has antiproliferative and prodifferentiation activities against myeloid leukemia and myeloma cell lines at clinically achievable concentrations [140,556,557]. Paricalcitol activity was dependent on the presence of VDR, as it was unable to induce differentiation of mononuclear bone marrow cells from VDR-KO mice, whereas cells from WT mice were differentiated toward monocytes/macrophages [140]. Furthermore, paricalcitol was able to inhibit tumor growth without causing hypercalcemia in immunodeficient mice [140].

### 7.2.4 Doxercalciferol

This is a synthetic  $1\alpha(\text{OH})\text{D}_2$  vitamin  $\text{D}_2$  analog that undergoes metabolic activation in vivo to form  $1\alpha,25$ -dihydroxyvitamin  $\text{D}_2$  ( $1\alpha,25(\text{OH})_2\text{D}_2$ ). It has been commonly used since 1999 for the treatment of secondary hyperparathyroidism, chronic kidney disease, and metabolic bone disease (reviewed in Refs. [550,558]). Doxercalciferol inhibited tumor growth in a xenograft model of retinoblastoma with less toxicity than  $1,25(\text{OH})_2\text{D}_3$  [559]. It also synergizes with other compounds and can induce cell death of hepatocellular carcinoma cells [560] and apoptosis of ALL [561] and AML cells [477] in vitro.

### 7.2.5 PRI-1906 and its derivatives

The vitamin  $\text{D}_2$  analogs (24E)-(1S)-24-dehydro-24a-homo- $1,25$ -dihydroxyvitamin  $\text{D}_2$  (PRI-1906) and (24E)-(1S)-24-dehydro-24a,26,27-trihomo- $1,25$ -dihydroxyvitamin  $\text{D}_2$  (PRI-1907) with side chains extended by one (C-25 dimethyl) and two (C-25-diethyl) carbon units, respectively, displayed markedly elevated antiproliferative and differentiation-inducing activity in AML cells compared with  $1,25(\text{OH})_2\text{D}_3$  [37,562]. PRI-1906 and PRI-1907 underwent further 19-nor modification of the A-ring resulting in the PRI-5201 and PRI-5202 analogs, respectively [563], or 24Z (24-cis) modification in the

extended side chains giving the PRI-1916 and PRI-1917 analogs [564]. The 19-nor modification of PRI-1906 and PRI-1907 greatly enhanced their potency against AML cells [549,565], while the 24-cis (24Z) modification had an opposite effect [546]. The potency of PRI-1916 was slightly higher or equal to that of PRI-1906, while PRI-1917 was significantly less potent than PRI-1907 [546]. While PRI-1906 is substantially less calcemic than  $1,25(\text{OH})_2\text{D}_3$ , the much stronger differentiation inducer PRI-1907 is also much more toxic [565]. Interestingly, the 19-nor derivative of PRI-1906 (PRI-5201) exhibited only a slightly elevated calcemic effect in mice compared with its precursor, whereas the 19-nor modification of PRI-1907 greatly reduced the calcemic activity of the resulting PRI-5202 analog [144,565].

If reproduced in other systems, these data will support the hypothesis that the anticancer and calcemic activities of VDDs are regulated separately and, perhaps, independently as a result of complex interactions between the A-ring and side-chain structural moieties of VDDs and the ligand binding domain (LBD) of the VDR [566,567].

### 7.2.6 Derivatives of lithocholic acid

It has been documented that VDR has an important role in detoxification of xenobiotics and some endogenous compounds in the organism [568]. In particular, VDR binds potentially toxic metabolites of bile acids, such as lithocholic acid (LCA), which can activate this receptor and induces transcription of selected genes, including that encoding the detoxifying enzyme CYP3A4. Interestingly, in contrast to  $1,25(\text{OH})_2\text{D}_3$ , LCA did not stimulate the expression of the intestinal calcium channel TRPV6 in mice, and thus did not induce hypercalcemia [569]. Whether a similar selectivity exists in humans should be further investigated, since LCA analogs were able to upregulate TRPV6 gene transcription in a human intestinal cell line [570]. Recently, some LCA analogs were reported to be potent inducers of cell differentiation, with relatively low calcemic activity [570–572].

## 7.3 Potential mechanisms by which vitamin D analogs have increased biological activity

It has been speculated that, when compared with  $1,25(\text{OH})_2\text{D}_3$ , synthetic vitamin D analogs have several potential advantages for the treatment of human disease and may be of clinical use [541,548,573,574]:

- structure-dependent dissociation between enhanced antiproliferative and differentiation-inducing effects and reduced calcemic activity;
- reduced affinity to the serum vitamin D-binding protein;



- decreased catabolism by 24-hydroxylase;
- retention of biological activities by metabolic products of VDDs;
- increased stability of the ligand–VDR complex;
- increased dimerization with RXR $\alpha$  associated with increased affinity for VDREs in the promoters of target genes;
- enhanced recruitment of the DRIP coactivator complex.

It is also possible that different VDDs activate different signaling pathways, but it is still not clear how this would be achieved. However, there are likely to be cell type–specific differences in these mechanisms, and none of the aforementioned VDD features has been firmly established.

## 7.4 VDDs in combination with other agents

The concentrations of VDDs required to inhibit proliferation and/or induce differentiation of neoplastic cells in culture have the potential toxicity *in vivo*. Thus, various attempts have been made to lower the effective doses of VDDs through their combinations with other compounds that may act synergistically or additively yet have an acceptable toxicity. Particularly, the idea of administering VDDs either as adjuvants or as integral part of conventional chemotherapy has long attracted much interest [556,575,576]. Initial studies demonstrated a marked synergistic induction of differentiation in AML cell lines by low concentrations of 1,25(OH) $_2$ D $_3$  combined with the synthetic glucocorticoid dexamethasone [577] or the vitamin A derivative ATRA [43]. Since that time, numerous reports have shown that VDDs are capable of cooperating with various agents in experimental models of cancer (reviewed in Refs. [576,578,579]), and in some clinical studies described in the following. Here, we describe VDD cooperation with cytotoxic and cytostatic drugs, differentiation inducers, inhibitors of signaling kinases, anti-inflammatory agents, and phytochemicals.

### 7.4.1 Cytotoxic and cytostatic drugs

A number of preclinical studies have shown that VDDs potentiate cytotoxicity of chemotherapeutic agents in various cancer cell types, including AML cells, whereas such cytotoxic and cytostatic drugs can enhance the differentiation-inducing activity of VDDs (reviewed in Refs. [576,578]). For instance, enhanced growth arrest and cytotoxicity was demonstrated using combinations of VDDs with 1- $\beta$ -D-arabinofuranosyl cytosine (AraC; cytarabine) and other cytotoxic drugs ([580,581]. Some studies have suggested that the sequence of administering the compounds may be important [580,582]. For example, pretreatment with

etoposide enhanced the subsequent differentiation-inducing action of 1,25(OH) $_2$ D $_3$ , but pretreatment with 1,25(OH) $_2$ D $_3$  had little effect on the cytotoxic activity of etoposide. The potentiating effect of etoposide was associated with upregulation of VDR both at the miRNA and protein levels [582].

Further, it was proposed that DNA damage-induced elevation of VDR levels in etoposide- and doxercalciferol-treated non–small-cell carcinoma and osteosarcoma cells is mediated by p73, but not by p53. VDR upregulation was accompanied by sensitization of the cells to 1,25(OH) $_2$ D $_3$  treatment [583]. Studies using AML cell lines and patient-derived blasts have shown that the combinations of 1,25(OH) $_2$ D $_3$  or doxercalciferol and the plant polyphenol carnosic acid (CA) added following AraC-treatment selectively increased its cytotoxicity to AML blasts, but not to normal bone marrow cells [471,472]. The enhanced cell death was associated with upregulation of VDR in cells with DNA damage [472] without enhancing AraC-induced generation of reactive oxygen species (ROS) [584].

Both 1,25(OH) $_2$ D $_3$  [585] and paricalcitol [556] potentiated the growth-inhibitory and cytotoxic effects of As $_2$ O $_3$  on AML cells [405]. This drug has been successfully used for the treatment of APL, particularly when combined with ATRA [586,587]. The antiproliferative effect of paricalcitol/As $_2$ O $_3$  combination on NB4 human APL cells and U937 cells was associated with a decrease in the levels of the VDD-metabolizing enzyme 24-hydroxylase (CYP24A1) [556]. In another study, 1,25(OH) $_2$ D $_3$  was found to enhance the apoptotic activity of nutlin-3a, an inhibitor of p53-MDM2 interaction, in AML cells expressing wild-type p53 (MOLM-13 and OCI-AML3) by suppressing antiapoptotic protein BCL-2 and p90RSK and by inducing the proapoptotic protein PIG-6 [28]. Several reports described the ability of 1,25(OH) $_2$ D $_3$  to cooperate with epigenetically active compounds, such as the demethylating agent 5-aza-2'-deoxycytidine [588] and the histone deacetylase (HDAC) inhibitor sodium butyrate [589]. These and studies described in the following exemplify the still not totally explored potential of combining VDDs with other compounds.

### 7.4.2 Retinoids

The fact that the VDR and the RXR $\alpha$  interact to form a heterodimeric transcription factor [590] suggests that VDDs and retinoids, both being potent differentiation inducers, can cooperate in producing anticancer effects. Indeed, synergistic or additive prodifferentiation and antiproliferative activities of 1,25(OH) $_2$ D $_3$  combined with ATRA or 9-cis retinoic acid (9-cis-RA) have been reported in various cancer cell types including AML cells (reviewed in Refs. [27,578]). In most studies, AML cells treated with 1,25(OH) $_2$ D $_3$ /retinoid combinations

displayed a monocytic phenotype, though granulocytic or mixed phenotypes were also described depending on the particular cell subtype and concentrations of each inducer (e.g., Refs. [578,591]). Monocytic differentiation of 1,25(OH)<sub>2</sub>D<sub>3</sub>/ATRA-treated myeloblastic AML cells or normal myeloid progenitors was concomitant with the direct repression of ATRA transcriptional activity by 1,25(OH)<sub>2</sub>D<sub>3</sub> [592]. This effect was attributed to the formation of dominant-negative VDR-containing complexes bound to the promoters of ATRA target genes, such as the retinoic acid receptor beta (RAR $\beta$ ) gene. In contrast, 1,25(OH)<sub>2</sub>D<sub>3</sub> potentiated ATRA-induced granulocytic differentiation in APL cells, and this was associated with the enhancement of ATRA-induced transactivation of the RAR $\beta$  promoter [593]. The combination of ATRA with 1,25(OH)<sub>2</sub>-16-ene-23-yne D<sub>3</sub> cooperatively decreased c-MYC expression [594]. Interestingly, U937 cells exposed to a moderate thermal stress responded with increased differentiation after the addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> and ATRA, suggesting that induction of heat shock protein may be sequestering a protein that may favor proliferation or differentiation [595]. The induction of M2 macrophage-like phenotype in AML cells by 1,25(OH)<sub>2</sub>D<sub>3</sub>/retinoid combinations has been reported [194]. The retinoids tested were 9-cis-RA, ATRA, or the synthetic RAR $\alpha$  agonist Am80. All these combinations promoted macrophage-like morphological changes and increased the expression of M2 macrophage markers, such as CD163, ARG1, and IL-10. RAR $\alpha$  agonist (AGN195183) was effective in driving neutrophil differentiation of NB4 cells, and this agonist synergized with a low concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> to drive monocyte differentiation of NB4 and KG1 cells [596].

#### 7.4.3 Protein kinase C activators

Other differentiation inducers, such as PKC activators 12-O-tetradecanoylphorbol-13-acetate (TPA) [597,598] and bryostatin-1 [599], were found to synergize with VDDs in the induction of AML cell differentiation and growth arrest. For instance, Bhatia et al. reported that the combination of 1,25(OH)<sub>2</sub>D<sub>3</sub>, TPA, and M-CSF resulted in a synergistic response in NB4 cells, causing a complete differentiation to fully functional adherent macrophages with a rapid arrest of cell growth in the first 24 h [597]. Using cDNA microarrays and Northern blot analyses, it has been shown that the synergistic induction of macrophage-like differentiation of myeloblastic leukemia cells ML-1 by TPA and the 1,25(OH)<sub>2</sub>D<sub>3</sub> analog KH1060 was associated with an enhanced expression of several TPA target genes. These included tryptase  $\beta$ 1, BCL2A1, ATF3, CD14, FBP1, and PrP [598]. This illustrates that PKC (TPA activated) and MAPK (VDD activated) can synergize for an optimal differentiation of a hematopoietic cell type [598].

#### 7.4.4 Antiinflammatory agents

VDDs have antiinflammatory activity [600,601]. Nonsteroidal antiinflammatory drugs (NSAIDs) enhance the differentiation of HL60 cells in response to 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs [602,603]. This effect may occur because of a block of NF- $\kappa$ B activation. Using a panel of AML cell lines and patient-derived blasts, it was shown that different COX1/2 inhibitors markedly potentiated 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced cell differentiation and G0/G1 cell cycle arrest through the RAF1-dependent mechanism [604]. It was also demonstrated that 5-lipoxygenase inhibitors can enhance 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation of HL60 cells in a p38 MAPK-dependent manner [605].

#### 7.4.5 Kinase inhibitors

Specific inhibitors of p38 MAPK (SB203580 and SB202190) were found to accelerate monocytic differentiation of HL60 cells induced by low concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> [603,606]. This augmented potency was associated with a prolonged activation of the JNK and, to a lesser extent, the ERK pathways. Recently, specific inhibitors of the MEK5/ERK5 MAPK signaling pathway were found to cooperate with VDDs, promoting a macrophage-like phenotype, enhancement of phagocytic activity, and growth inhibition in AML cells in vitro and ex vivo [33,50]. This was associated with the upregulation of the M-CSFR and was not seen when M-CSFR expression was knocked down. Inhibition of glycogen synthase kinase-3 (GSK3), e.g., by SB415286, potentiated the differentiation response of AML cell lines to low concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> and augmented the ability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to induce cell growth inhibition in vitro and in vivo [607]. Mechanistic studies revealed that GSK3 inhibition increased the transcriptional activity of VDR, which resulted from receptor phosphorylation leading its enhanced coactivator, SRC-3 [607]. Inhibition of mTORC1 by the rapamycin analog everolimus potentiated the growth-inhibitory and differentiation-inducing effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in U937 cells both in vitro and in vivo [181]. The addition of everolimus enhanced 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated transcriptional activation of p21<sup>Cip1</sup> expression, which apparently was associated with increased levels of VDR bound to the p21<sup>Cip1</sup> (CDKN1A) promoter.

#### 7.4.6 Phytochemicals

A number of phytochemicals (aka “botanicals”) have been reported to enhance differentiation of AML cells induced by VDDs (reviewed in Ref. [578]). Among these are the plant polyphenolic antioxidants carnosic acid (CA), curcumin, and silibinin [473,608,609] and sesquiterpene lactones [610]. The potential therapeutic

significance of such combinations was supported by the findings that CA preparations and low calcemic VDDs synergistically inhibited leukemia progression in mouse models [611,612]. The mechanisms underlying the aforementioned cooperative effects involve modulation of various cellular systems. Thus, CA has been shown to upregulate VDR/RXR $\alpha$  expression and functional activity, which may explain the increased sensitivity of cancer cells to lower doses of VDDs [473]. These effects were mediated by the activation of the NRF2/antioxidant response element (NRF2/ARE) signaling pathway and AP-1 transcription factor [143,613]. Based on these findings, it was demonstrated that other structurally distinct NRF2 activators, including dimethyl fumarate (Tecfidera) approved for the treatment of multiple sclerosis, were capable of synergistically enhancing VDD-induced differentiation of AML cells. Further, combined treatment with DMF and the highly potent vitamin D<sub>2</sub> analog PRI-5202 cooperatively inhibited leukemia progression in a xenograft model of AML in vivo [144].

Interestingly, silibinin was found to modulate the differentiation effects of VDDs in a cell type-dependent manner. While enhancing the differentiation in the HL60 and OCI-AML3 cell lines and leukemic blasts from some patients with AML, it had no effect or even inhibited the differentiation of the MOLM-13 and U937 cell lines and primary blasts from other patients [191,404,613,615], which may reflect the different mutations that are present in these cells. The positive influence of this polyphenol was associated with the upregulation of transcription factors of the JUN and C/EBP families [614], whereas its negative effects with the inability to activate NRF2/ARE as well as with downregulation of RXR $\alpha$  expression [613] and activation of the oncogenic COT1/TLP2 and ERK5 kinases [191]. Other signaling kinases, such as ERK1/2 and JNK MAPK and PKC [259,609], were shown to positively contribute to the differentiation-enhancing effects of various phytochemicals. On the other hand, the tumor suppressor p53 status in AML cells did not affect the differentiation-associated G1 cell cycle arrest induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> combined with CA or silibinin [404], while miRNA-181a was found to antagonize doxercalciferol/CA-induced cell differentiation and cell cycle arrest [615]. In addition, combinations of three agents, including 1,25(OH)<sub>2</sub>D<sub>3</sub> and either the p38 inhibitor SB202190 plus CA [616] or the COT1/TLP2 inhibitor 4-(3-chloro-4-fluorophenylamino)-6-(pyridin-3-yl-methylamino)-3-cyano-[1,2,3,4,5,6,7]-naphthyridine plus silibinin [191] further potentiated the antileukemic activity against AML cells and primary AML blasts.

In summary, treatment of leukemia or MDS with a VDDs is unlikely, by itself, to be successful, but when given either in the maintenance phase of therapy after the leukemic patient is placed into remission or

combined with other agents, vitamin D compounds may be useful therapeutically. Furthermore, 1,25(OH)<sub>2</sub>D<sub>3</sub> can induce the expression of the antimicrobial peptide CAMP [617], which may afford the cancer patient some protection from life-threatening infections while receiving aggressive chemotherapy.

## 7.5 Clinical trials of VDDs in hematopoietic malignancies

As discussed before, encouraging results of numerous preclinical studies with VDDs, alone and in combination, against AML cells provided the basis for clinical trials of these agents. However, the attempts to utilize the prodifferentiation and antiproliferative properties of VDDs in clinic have had so far minimal success, possibly due to the variable levels of VDRs in the malignant cells, the heterogeneity of AML subtypes, and sub-optimal design of clinical trials (reviewed in Refs. [618–620]). More trials of VDDs in hematological malignancies are being conducted (examples are shown in Table 86.4), but outcomes at this time are unclear. Despite several examples of promising clinical efficacy of VDDs, there are three major concerns: (1) The lack of definition of a sensitive target subset of AML patients because clinical trials conducted so far have generally used extremely heterogeneous patient populations and, in many cases, small numbers of patients, often without controls. (2) The still unknown optimal choice of a vitamin D analog and the dosing schedule. (3) No coherent mechanistic basis for the claimed improved therapeutic window. Further, since different dosage and time schedule of compound administration were used, it is not possible to directly compare the VDD effects on the diseases studied. The results of clinical studies of several VDDs are provided below.

### 7.5.1 1,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub>

Treatment of elderly MDS patients having moderate/severe anemia with the combination of erythropoietin with 1,25(OH)<sub>2</sub>D<sub>3</sub> and 13-cis retinoic acid resulted in a long-lasting (7 years) erythroid response in the majority of MDS patients with unfavorable prognostic features for response to erythropoietin alone [624]. In patients with MDS and AML treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> in combination with cytarabine, 6-thioguanine, and 13-cis-retinoic acid, the response rate was 50%, with 27% complete remission [625]. The combination of 1,25(OH)<sub>2</sub>D<sub>3</sub>, AraC, and hydroxyurea resulted in complete or partial responses in 79% of elderly patients with AML [626]. These regimens had acceptable toxicity. A recent study evaluated a 4-year maintenance therapy with low-dose cytotoxic agents combined with 1,25(OH)<sub>2</sub>D<sub>3</sub> and 13-cis retinoic acid in poor-prognosis

**TABLE 86.4** Selected clinical trials of VDDs in hematological malignancies during the years 2015–21.

Study number	Condition/disease	Compound	Aim of the study	Age group; enrolment	Phase	Status
NCT02802267	AML	I-cal	Efficacy study of I-cal in combination with decitabine in patients unfit for standard chemotherapy	65–75; 110	II	O
NCT03176849	HSCT	25(OH)D <sub>3</sub>	Safety and efficacy of a single, high dose of oral vitamin D; the start of transplant followed by maintenance supplementation in children undergoing HSCT	<3–>12; 49	IV	C <sup>a</sup> [621,622]
NCT00068276	MDS	25(OH)D <sub>3</sub>	Efficacy in terms of hematological improvement, in patients with low- or intermediate-risk of MDS (the effect on disease symptoms, fatigue, and the overall health-related quality of life)	Any; 36	II	C
NCT00104806	MDS	25(OH)D <sub>3</sub>	Determination of the complete response rate and the rate of hematological improvement in patients with MDS treated with arsenic trioxide and 25(OH)D <sub>3</sub>	Any; 26–60	II	T
NCT00064376	MDS	P-cal	Determination of the clinical effects of P-cal in patients with MDS; its influence on the decrease the risk of development of leukemia without causing undue toxicity	>25; 20	II	C
NCT01787409	DLBCL/CML	25(OH)D <sub>3</sub>	The role of 25(OH)D <sub>3</sub> in improving survival in patients with newly diagnosed cancer and vitamin D insufficiency	>18; 713	N/A	O <sup>a</sup> [623]
NCT01521936	AML	25(OH)D <sub>3</sub>	Partially randomized studies on the side effects, administration route, and dosage of 25(OH)D <sub>3</sub> in AML patients undergoing intensive induction chemotherapy	>18; N/A	II	T
NCT02341495	AML	25(OH)D <sub>3</sub> Deferasirox Azacytidine	Study on a novel therapeutic combination of deferasirox, 25(OH)D <sub>3</sub> , and azacytidine in older patients with newly diagnosed AML who are unfit for standard chemotherapy or HSCT	>65; N/A	II	T
NCT02949570	CML	I-cal	To assess the efficacy of I-cal in combination with imatinib in CML patients with molecular residual disease on imatinib monotherapy	>18; 54	II	O
NCT00052832	MDS/CML	D-cal	Determination of the response rate, toxicity profile, and influence on overall survival	>18; 41	II	C

<sup>a</sup>Results published.

1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>; D-cal, doxercalciferol; DLBCL, diffuse large B-cell lymphoma; I-cal, inecalcitol; P-cal, paricalcitol; AML, acute myeloid leukemia; CML, chronic lymphoid leukemia; CLL, chronic lymphocytic leukemia; HSCT, hematopoietic stem cell transplant; MDS, myelodysplastic syndromes; C, completed; T, terminated; O, ongoing; N/A, not applicable.



elderly patients with AML and MDS [627]. This treatment resulted in a lower relapse incidence and a longer disease-free and overall survival compared with the control patients who did not receive maintenance treatment.

A combination treatment of elderly patients with AML with the 1,25(OH)<sub>2</sub>D<sub>3</sub> precursor 25(OH)D<sub>3</sub> and the iron-chelating agent deferasirox resulted in a significant increase in median survival in comparison with patients receiving best supportive care alone. Interestingly, the only factor associated with survival benefit was the serum 25(OH)D<sub>3</sub> level prior to treatment. Thus, the median overall survival of patients with normal 25(OH)D<sub>3</sub> levels ( $\geq 50$  nmol/L) was significantly higher compared with the group with what the authors considered to be vitamin D deficiency ( $\leq 50$  nmol/L) [628]. This study was based on the previous findings by this group [629] showing that combination of iron depletion and 1,25(OH)<sub>2</sub>D<sub>3</sub> enhanced the differentiation of both HL60 cells and AML primary blasts in vitro and suppressed the growth of HL60 xenografts in mice. Importantly, several other studies have also demonstrated that low levels of circulating 25(OH)D<sub>3</sub> were associated with shorter survival, worse prognosis, and adverse outcomes in newly diagnosed and treated patients with MDS [630], AML [630,631], CLL [632], and follicular lymphoma [536].

### 7.5.2 Synthetic vitamin D analogs

Several VDDs have been studied as potential therapy for patients with MDS, leukemias, and lymphomas, as described in the following.

#### 7.5.2.1 Alfalcidol

Among the early attempts, beginning in the 1980s, to demonstrate the therapeutic value of VDDs were Japanese studies of alfalcidol, an analog that only requires 25-hydroxylation in vivo to promote VDR activation (e.g., Ref. [633]). Leukemic transformation-free survival was monitored in patients with MDS treated with 4–6 µg/day alfalcidol (per os) for 17 months or placebo [633]. An improvement of hematologic parameters was detected in only one patient, though leukemia-free survival and AML incidence of the treated group had significant advantage over the control group. These results suggested that oral administration of alfalcidol may prevent the progression of MDS to overt leukemia. A multicenter prospective phase II clinical trial of vitamin K2 (approved in Japan for osteoporosis treatment [634] and shown to ameliorate cytopenia in MDS [635]) and its combination with oral alfalcidol (0.75 µg daily) was conducted for 16 weeks in patients with low-risk MDS [636]. The results demonstrated that the combination was more effective than vitamin K2 alone in improving anemia and thrombocytopenia. In a small-scale pilot study, patients with low-grade

non-Hodgkin's lymphoma were treated with 1 µg oral alfalcidol daily [637]. Patients with the follicular small, cleaved, subtype achieved complete or partial remission, whereas none of patients with small lymphocytic lymphomas responded. This analog is currently rarely studied in relation to malignant diseases.

#### 7.5.2.2 Inecalcitol

Inecalcitol has been tested in a phase II trial in patients with CLL at a daily dose of 2 mg. The results demonstrated that 52% of patients experienced stabilization or decrease of blood leukemic lymphocytes counts [638]. An exploratory phase II study of inecalcitol (4 mg daily) is ongoing in older CML patients under treatment with imatinib (EU Clinical Trials Register: 2014–004347-12). In addition, a randomized phase II efficacy trial of inecalcitol in combination with decitabine has been initiated in older AML patients unfit for standard chemotherapy (NCT02802267).

### 7.6 Paricalcitol and doxercalciferol

Small-scale clinical trials of oral paricalcitol [639] and doxercalciferol [640] were conducted in MDS patients. Although paricalcitol was well tolerated in all patients at doses of 8–56 µg/day, it had only minimal activity against MDS [639]. Other clinical trials have demonstrated that it has reduced calcemic activity [641,642]. Patients who received 12.5 µg/day doxercalciferol, for 12 weeks, did not develop hypercalcemia, but they also did not obtain a clinical response [640].

## 8. Significance of basic and clinical studies of VDDs in hematopoietic malignancies: from bench to bedside

As discussed before, the encouraging results of numerous preclinical studies of VDDs alone and in various combinations against hematopoietic malignancies provided the basis for clinical studies and formal clinical trials of these agents.

There are numerous reports of an association between suboptimal levels of 25(OH)D<sub>3</sub> and the prevalence of hematopoietic malignancies, including monoclonal gammopathies such as multiple myeloma (MM) [643–646]. Vitamin D deficiency also appears to predict adverse outcome, such as poor overall patient survival, in several hematopoietic malignancies, including MM [647], intensively treated adult AML [631], MDS and secondary oligoblastic AML [630], as well as follicular lymphoma [536].

Supporting these associations, several examples of potentially promising clinical efficacy of VDDs in hematopoietic malignancies have been published. The

ex vivo cytotoxic activity of myeloma-associated macrophages can be enhanced by vitamin D supplementation [648], and VDR function is necessary for the inhibition of growth of all plasmablastic cell lines that were studied [649]. This may also explain that VDR polymorphisms are associated with several cancers including MM, and 25(OH)D insufficiency can be demonstrated in those individuals [650–652].

Several, more direct, clinical studies showed that supplementation of MM patients with VDDs can positively influence the prognosis of monoclonal gammopathies. For example, the administration of cholecalciferol (approximately 10,000 IU/week) significantly increased the patients' hemoglobin levels, the erythrocyte and leukocyte counts [643]. Oral 1,25(OH)<sub>2</sub>D<sub>3</sub> supplementation (0.25 µg TID for 30 days) of patients with MM and Hodgkin's and non-Hodgkin's lymphoma following autologous stem cell transplantation significantly shortened lymphocyte count recovery, and the relapse-free survival was significantly better [494]. However, the attempts to utilize the prodifferentiation and antiproliferative properties of VDDs in the clinic have had so far minimal success, possibly due to the variable levels of VDRs in the malignant cells, the heterogeneity of the subtypes of AML and other malignant hematopoietic disorders, and suboptimal design of clinical trials including the dosage of VDDs used (reviewed in Refs. [618–620]). By August 16, 2021, an impressive number (3471) of clinical studies with vitamin D<sub>3</sub> or its analogs were recorded in the [clinicaltrials.org](https://clinicaltrials.org) database, and 447 of these studies were at that time recruiting patients (examples are shown in Table 86.4) [3,621,623,653]. Of note, few of the terminated trials had potentially positive outcomes, and many were closed because of poor patient accrual or loss of financial support. However, as a number of trials are still recruiting patients, positive outcomes cannot yet be excluded ([www.clinicaltrials.gov](https://www.clinicaltrials.gov)). Further, since various dosages and schedules of compound administration were tested in different trials, there is still no agreement on the safe dosages of VDDs that exhibit anticancer properties and do not cause hypercalcemic side effects, and it is not possible to directly compare the VDD effects on the diseases studied.

But before abandoning all hope for the potential of VDDs in the management of hematopoietic malignancies, the clinical trials design needs to be improved. The main problem with the inconspicuous results of clinical trials that have been terminated is likely that concentrations of the VDDs used have been too low. There is now a slowly increasing realization among health providers that the maximal dosage of VDDs for producing antineoplastic effects is much higher than the concentrations needed for its bone health effect, but this realization needs to reach the regulatory

agencies. Thus, if frequent determinations of patients' serum calcium levels can be factored into the trial protocol, there seems to be no reason that at least 4000 IU of vitamin D daily should be a part of the treatment cocktail. Such off-label use of vitamin D may be helpful in compassionate situations, but the analysis and the publication of the results are likely to be problematical [27,630,631].

## 9. Conclusions

It is clear that the physiological forms of vitamin D have important roles in human health, including the development of the normal hematopoietic system, and the still not totally fulfilled promise as a tool to combat hematopoietic malignancies. Regarding the latter, the search for VDDs that dissociate calcium mobilizing properties in vivo from differentiation-inducing, antiproliferative, or cell death-enhancing properties continues. However, it seems likely that the combinations of existing low-calcemic analogs with nontoxic compounds, which potentiate vitamin D–derived antineoplastic effects, may reach the clinic sooner than the new analogs. Yet, both of these will require greater investment of effort and funding than has recently been available. Nonetheless, the hematopoietic cells appear to be well poised to provide exciting results necessary to further increase the credibility to vitamin D and cancer field.

## 10. Summary points

- VDDs are powerful differentiating agents that target diverse cell types, but the physiological role of 1,25(OH)<sub>2</sub>D<sub>3</sub> in development remains to be fully determined.
- The presence of VDR is strictly required for VDD-induced monocyte/macrophage differentiation of AML cells.
- VDDs induce time- and cell type–dependent modulation of myeloid leukemia cell growth, with an initial proliferative burst followed by cell cycle arrest, and generally increase cell survival.
- The immunomodulatory role of 1,25(OH)<sub>2</sub>D<sub>3</sub> is beneficial in leukemias and in acute infections.
- Clinical trials of 1,25(OH)<sub>2</sub>D<sub>3</sub> at concentrations that do not cause hypercalcemia are unlikely to have any therapeutic effect in leukemia patients.
- The complex relationships between the actions of VDDs on differentiation, the retardation of cell cycle progression, and cell survival of leukemic blasts require intensive study.

- An understanding of the complex actions of VDDs should allow a rational design of agent combinations that synergize with VDDs for clinical trials of therapy of MDS and leukemia.

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# Vitamin D, inflammation, and cancer

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## OBJECTIVES

- To review the main characteristics of the inflammatory process and its association with cancer.
- To understand how vitamin D prevents the development and progress of chronic inflammation.
- To understand the role of vitamin D in the anti-inflammatory actions of the immune system.
- To review the role of vitamin D in the most common inflammatory diseases.
- To understand which major molecular pathways of inflammation-associated cancers are modulated by vitamin D.
- To present concrete examples of how vitamin D affects the major inflammatory and cancer-promoting pathways in the intestines, prostate, breast, pancreas, and liver.

## 1. Introduction

Cancer arises due to accumulation of genetic changes that lead to acquisition of so-called hallmarks of cancer: self-sufficient proliferation, insensitivity to anti-proliferative stimuli, resistance to apoptosis, unlimited replicative and angiogenic potential, and the ability for invasion and metastasis [1]. Genomic instability and inflammation foster the acquisition of these hallmarks. The link between cancer and inflammation is based on

the observations that tumors often develop at sites of chronic inflammation and that inflammatory cells are found in the tumor tissue. In addition, the immune system affects cancer development through both pro- and anti-tumorigenic mechanisms, as recently reviewed by Greten and Grivnickov [2].

The inflammatory process is the response of the host to microbial infection or to tissue injury and is responsible for maintaining tissue homeostasis. Controlled inflammation protects against infection and tissue damage. Uncontrolled inflammation leads to autoimmunity and the development of multifactorial diseases, such as type 2 diabetes, inflammatory bowel disease (IBD), neurodegenerative diseases, asthma, chronic rheumatic disorders, and cancer [3]. Numerous chronic inflammatory diseases, such as pancreatitis, colitis, and hepatitis, are recognized risk factors for cancer of the pancreas, colon, and liver. Chronic inflammation has been linked to higher risk of developing cancers such as bladder, esophageal, ovarian, prostate, or thyroid cancer [4]. Inflammatory cells and mediators of inflammation are present in the environment of most tumors, irrespective of the etiology of the tumor. Moreover, in some types of cancer, oncogenic changes may create an inflammatory microenvironment that promotes the development of tumors [3].

The main function of vitamin D is to regulate calcium and phosphate homeostasis [5]. However, outside of mineral ion homeostasis, it also modulates the adaptive and innate immune system [6–8] (see also Chapters 94–96) and has cancer-preventive and therapeutic actions in an endocrine, paracrine, and autocrine fashion [9,10]. Therefore, it could become an ideal preventive/therapeutic agent in inflammation-associated cancer.



This chapter systematically explores the role of vitamin D in inflammation-associated cancer, focusing specifically on cancers of the colon, prostate, breast, pancreas, and liver.

## 2. Vitamin D and the vitamin D system

Vitamin D<sub>3</sub> is synthesized in the skin from 7-dehydrocholesterol after exposure to UVB radiation or is obtained through the diet. Vitamin D<sub>3</sub> is subsequently transported to the liver by the vitamin D-binding protein (DBP), where it is converted into its main circulating form 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) through hydroxylation on position C-25 by cytochrome P450 (CYP) enzymes with 25-hydroxylase activity [11]. Generally, 25(OH)D<sub>3</sub> concentrations lower than 50 nmol/L are associated with vitamin D deficiency, and serum levels below 75 nmol/L 25(OH)D<sub>3</sub> are indicators of vitamin D insufficiency [12]. 1,25-Dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), the most active vitamin D metabolite, is synthesized from 25(OH)D<sub>3</sub>, through 1 $\alpha$ -hydroxylation by 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase (CYP27B1) mainly in the kidney, but also in other tissues. 1,25(OH)<sub>2</sub>D<sub>3</sub>, bound to its cognate receptor, the transcription factor vitamin D receptor (VDR), regulates a plethora of different cellular processes [10,13]. VDR is expressed ubiquitously in most human and animal tissues, rendering these tissues as potential targets for 1,25(OH)<sub>2</sub>D<sub>3</sub>. For a more detailed description, see [Chapters 10–13](#).

Apart from its classical role in calcium metabolism and in bone health, 1,25(OH)<sub>2</sub>D<sub>3</sub> is involved in the regulation of numerous signaling pathways [14,15]. Its activity correlates with the induction of differentiation in normal cells and inhibition of proliferation, angiogenesis, activation of apoptosis, repression of invasion, and metastasis in tumor cells, resulting in inhibition of tumor growth and even in tumor regression [9,16–18].

One of the best understood extra-skeletal functions of vitamin D is its immune-regulatory function. Vitamin D has also antimicrobial and anti-inflammatory effects [19] (see [Chapter 94](#)). 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of specific genes of the innate immune system, such as cytokines, pattern recognition receptors, defensins, and other antimicrobial peptides. It also regulates the adaptive immune system by modulating the activity of T cells [20,21] (see [Chapter 96](#)). The capacity of vitamin D to inhibit inflammation also contributes to its anti-tumorigenic effect, as chronic inflammation is a major determinant of several cancers [22], as mentioned before. Vitamin D has anticancer properties in a large variety of cancers, being most effective in colorectal [23–26] ([Chapter 89](#)), breast [27–29] ([Chapter 88](#)), and prostate cancers [30–32] ([Chapter 91](#)). In some cancers,

aberrant expression of components of the vitamin D system may impede the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Numerous preclinical studies support the association between vitamin D supplementation and prevention from the development and progression of different malignancies [16,33,34]. However, human clinical trials are still inconsistent, and the resulting data are incomplete and controversial [13,35–38].

## 3. Inflammation

Inflammation is a very complex process that accompanies a severe disturbance in tissue homeostasis as result of multiple different causes such as infection, stress, smoking, obesity, or exposure to mechanical or chemical contaminant agents that trigger the activation of the innate and adaptive immune system. Under physiological conditions, inflammation is a protective, self-limiting response to infectious agents and to tissue injury. Its main role is elimination of pathogens and the support of tissue regeneration [39]. Chronic inflammation is considered the “common soil” for multifactorial diseases, such as asthma, type 2 diabetes, rheumatic disorders, and several cancers [3,22,40]. Indeed, inflammation is frequently involved in tumor progression—therefore, Hanahan and Weinberg [1] added inflammation to the hallmarks of cancer.

### 3.1 Main characteristics of inflammation

The inflammatory response has four main components: (1) the inducers of inflammation (e.g., bacteria, viruses, irritants such as asbestos, chemicals, etc.), (2) the sensors recognizing inflammation (e.g., Toll-like and NOD-like receptors), (3) the mediators of inflammation (e.g., pro-inflammatory chemokines, cytokines, prostaglandins [PG]), and (4) the target tissues [39].

Some of the viral and bacterial inducers of inflammation are considered to be associated with 15%–25% of the cancers worldwide, e.g., cervical and hepatocellular carcinoma, or some forms of gastric cancer [41]. The sensors and mediators of inflammation are also important constituents of the tumor microenvironment. In some cancers, the inflammation precedes the malignant transformation (extrinsic process), while in other types of cancer, oncogenic changes induce the inflammatory environment that promotes tumorigenesis (intrinsic process) [42]. High levels of inflammatory cytokines in tumors are also indicators of poor prognosis.

The acute and chronic inflammatory response is driven by different subpopulations of immune cells. The innate immune system (monocytes, macrophages, dendritic cells, mast cells) is responsible for the acute

response. Its failure to eradicate the acute inflammation leads to chronic inflammation, a process involving the adaptive immune system: the T and B lymphocytes. The T lymphocyte population consists of several subgroups, comprising the cytotoxic CD8<sup>+</sup> T cells, T helper cells (Th), regulatory T cells (Treg),  $\gamma\delta$  T cells, memory cells, and natural killer cells [8]. The Th cells are considered either pro-inflammatory (Th1 or Th17) or anti-inflammatory (Th2) [2,43]. Tregs reduce proliferation of the effector cells and are involved in tolerogenic processes [2,8].

In most tissues, a few major pathways (such as NF- $\kappa$ B, STAT3, COX-2/PGE<sub>2</sub>, Wnt/ $\beta$ -catenin) orchestrate the inflammatory process. Inappropriately activated nuclear factor  $\kappa$ B (NF- $\kappa$ B) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) are considered as major mediators of inflammation [3,44]. Inflammatory cytokines secreted by the immune cells or the inflamed cells activate the transcription factors NF- $\kappa$ B and signal transducer and activator of transcription 3 (STAT3) in both the immune cells and epithelial tissue. NF- $\kappa$ B and STAT3 play an important role also in tumorigenesis, among others, by upregulation of the cyclooxygenase 2 (COX-2)/prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) signaling. COX-2/PGE<sub>2</sub> signaling is often deregulated in inflammation-associated tumorigenesis [45,46], as are the p38 and the stress-induced kinase pathways (ERK/MAPK) [19]. Wnt/ $\beta$ -catenin signaling maintains tissue homeostasis and is responsible for tissue regeneration after damage [47,48]. This pathway is deregulated in ~53% of IBD patients and is one of the most frequently mutated pathways in sporadic colorectal tumors [49].

### 3.2 Vitamin D and inflammation

Studies conducted on cell lines and observational studies demonstrated the inhibitory role of vitamin D in the inflammatory process; however, the clinical trials to confirm the correlation between vitamin D and inflammatory markers are still inconsistent [50–52].

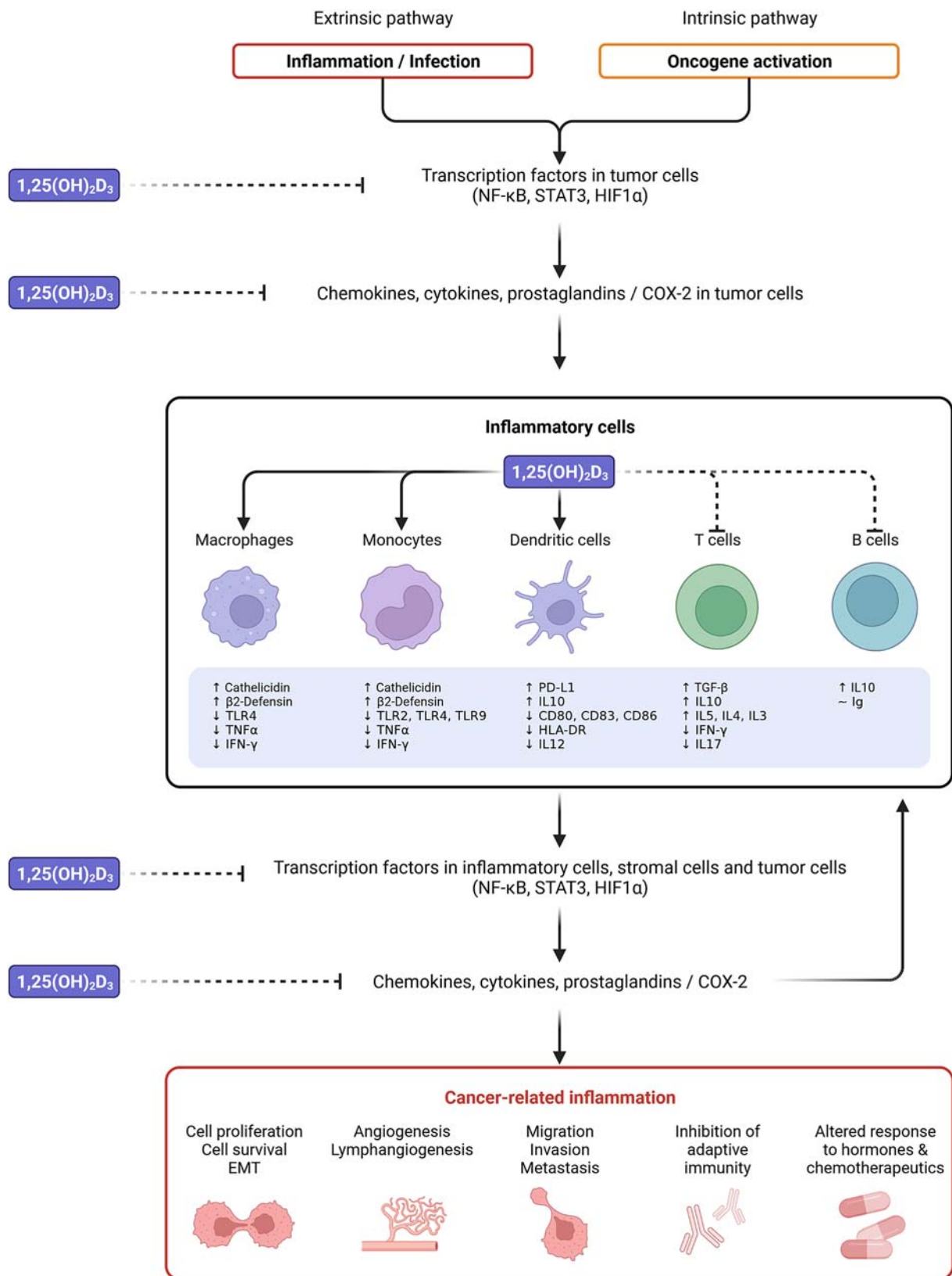
VDR and the vitamin D metabolizing enzymes are expressed in both the cells of the innate and the adaptive immune system, such as dendritic cells, monocytes/macrophages, in T and B cells, and in the cells of the target tissues (see Fig. 87.1 and also Chapters 94–96). Cells of the immune system produce 1,25(OH)<sub>2</sub>D<sub>3</sub> that exerts immunomodulatory effects, regulating innate and adaptive immune responses in a cell type-dependent and species-dependent manner [20,53]. Vitamin D and its metabolites are important players in regulation of different inflammation-related molecular pathways [54,55]; they affect all four components of the inflammatory response.

#### 3.2.1 Effect of vitamin D on inducers of inflammation: antimicrobial effects of vitamin D

Although the beneficial effect of sun for patients with different infections—both viral and bacterial—was recognized in the past, it was only recently that the molecular mechanism behind the observed antimicrobial effects of vitamin D and the role of the vitamin D target gene cathelicidin was elucidated, at least partially [56–58]. The human cationic antimicrobial protein (hCAP-18), belonging to the cathelicidin family of antimicrobial proteins, was identified first in 1995 [59]. hCAP-18 has broad antimicrobial activity against several pathogens, e.g. *Mycobacterium tuberculosis* [57] and *Staphylococcus aureus* [60]. One of the mechanisms of action of hCAP-18 is to trigger the activation of autophagy [61]. During infections caused by pathogens, 1,25(OH)<sub>2</sub>D<sub>3</sub> increased the antimicrobial capacities of antigen presenting cells—macrophages and monocytes, as well as of epithelial cells of the lung and intestines, placental trophoblasts, and keratinocytes, by inducing hCAP-18 expression [57]. In the monocyte/macrophage lineage, interferon gamma (IFN- $\gamma$ ) stimulated antimicrobial activity by inducing phagosome maturation, autophagolysosomal fusion, and autophagy through a 1,25(OH)<sub>2</sub>D<sub>3</sub>-dependent pathway [62]. 1,25(OH)<sub>2</sub>D<sub>3</sub> activated phagocytosis in monocytes and macrophages of pulmonary tuberculosis patients, by upregulating mannose receptor and the expression of autophagy genes ATG5 and BECN1 [63].

Differentiation of human monocytes toward dendritic cells under the influence of 1,25(OH)<sub>2</sub>D<sub>3</sub> and phenylbutyrate enhanced antimicrobial properties of the dendritic cells by stimulating induction of cathelicidin expression and ROS levels, thus increasing their capacity of killing *Staphylococcus aureus* [64]. In addition, patients with infectious diseases often have inadequate vitamin D levels. Chronic hepatitis C virus (HCV) infection was associated with vitamin D deficiency [65]. Chronic hepatitis B (CHB) patients with vitamin D deficiency had significantly higher HB virus replication than CHB patients with normal 25(OH)D<sub>3</sub> levels [66] (see Chapter 95). Moreover, vitamin D supplementation reduced disease severity and mortality from and infection with SARS-2/COVID-19 [67–70] (see also Chapter 99).

Not only does vitamin D affect microorganisms, but also the microorganisms affect expression, localization, and activity of VDR and the vitamin D system. In human fibroblasts the cytomegalovirus (CMV) downregulated VDR and CYP24A1 expression and upregulated CYP27B1 mRNA levels, inhibiting the antiviral effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> [71,72]. Colonization of colon cancer cells with *Salmonella typhimurium* upregulated VDR



**FIGURE 87.1** Effects of 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) on the inflammatory cells and cancer-related inflammation. Both extrinsic (represented by inflammation or infection) and intrinsic pathways (activated by genetic events such as activation of oncogenes) result in the activation of transcription factors in tumor cells that stimulate the production of proinflammatory chemokines, cytokines, and prostaglandins.

expression and transcriptional activity. Commensal enteric bacteria influenced VDR distribution in the colonic crypt [73].

### 3.2.2 Effect of vitamin D on receptors and mediators of inflammation

Vitamin D modulates the expression of receptors and mediators of inflammation (Fig. 87.2) in a complex, and cell- and tissue-dependent manner. Treatment of human keratinocytes with  $1,25(\text{OH})_2\text{D}_3$ -induced expression of CD14, a coreceptor of TLR4, and of the Toll-like receptor TLR2 [74], involved also in autophagy [75]. In monocytes,  $1,25(\text{OH})_2\text{D}_3$  suppressed expression of TLR2, TLR4, and TLR9 and TLR9-dependent upregulation of IL6 production, confirming the beneficial role of vitamin D supplementation in patients with autoimmune diseases with elevated IL6 serum levels [76].  $1,25(\text{OH})_2\text{D}_3$  inhibited the production of the proinflammatory cytokines IL17, IL21, and IFN- $\gamma$  and was associated with high expression of CTLA-4 and FoxP3 and upregulation of IL10, stimulating a regulatory response in T cells [77].

VDR inhibits NF- $\kappa$ B activity on multiple levels (Figs. 87.2 and 87.3). It was shown to prevent phosphorylation of the NF- $\kappa$ B subunit p65 and to inhibit its nuclear translocation by binding directly to p65 [73]. VDR prevented the phosphorylation of the inhibitor of NF- $\kappa$ B (I $\kappa$ B) and the release of NF- $\kappa$ B from I $\kappa$ B inhibition by binding to I $\kappa$ B kinase- $\beta$  (IKK $\beta$ ) [78].  $1,25(\text{OH})_2\text{D}_3$  extended the half-life of IKK $\beta$  mRNA [79] and inhibited COX-2 expression and subsequent PG synthesis in a dose-dependent manner [80]. Vitamin D stimulated the synthesis of antiinflammatory cytokines such as IL10 and TGF $\beta$  as well. IL10, released by Th2 cells, B cells, and macrophages, prevents host immune response to pathogens, while TGF $\beta$  is a mediator of the immunosuppressive function of Tregs [81,82].

### 3.2.3 Effect of vitamin D on immune cells

CD4<sup>+</sup> T cells of mice fed with  $1,25(\text{OH})_2\text{D}_3$ -supplemented diet had low levels of IL17, and the ability of the dendritic cells to mediate T cell transformation toward Th17 phenotype was reduced [83]. In nonobese diabetic mice,  $1,25(\text{OH})_2\text{D}_3$  suppressed proinflammatory TNF $\alpha$ , iNOS, and IL12p40 expression and inhibited the expression of chemokines with T cell recruiting properties, such as CXCL9, CXCL10, and CXCL11, in an IL10-dependent manner [84].

Vitamin D is able to regulate both the innate and the adaptive immune system (Fig. 87.1), although its primary role is strengthening the immediate response to infections. It enhances the antipathogenic actions of the innate immune system and regulates proliferation and differentiation of the adaptive immune system (see more detailed information in Chapters 94–96). The effect of vitamin D on immune cells is very complex, depending on the activation status of the immune cells [85]. VDR-bound  $1,25(\text{OH})_2\text{D}_3$  affects gene transcription either directly or indirectly, by modulating the epigenome [86].

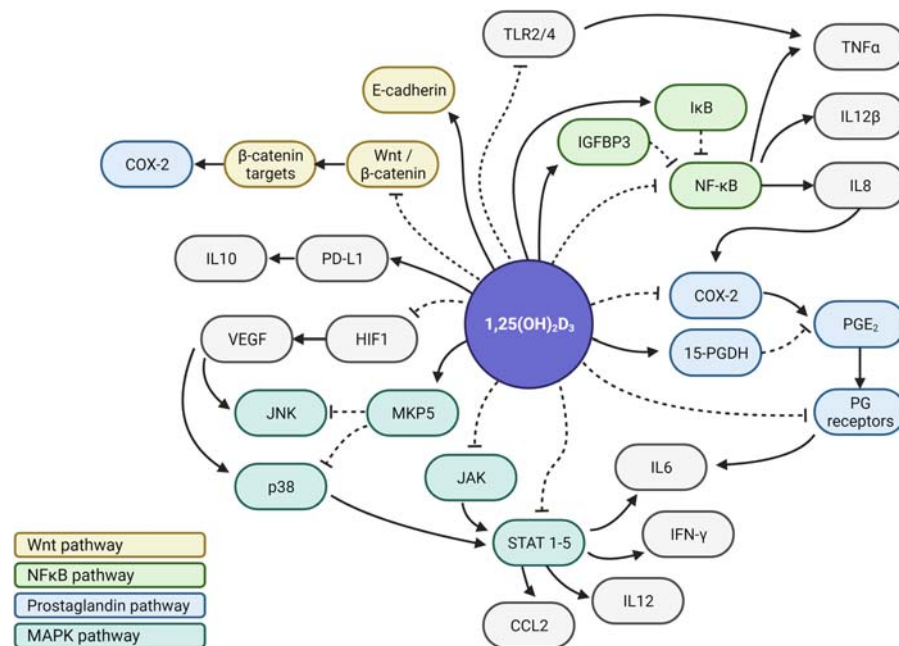
Naïve T cells have very low levels of VDR, but upon activation of the T cells, VDR is upregulated [51]. Both the systemic  $1,25(\text{OH})_2\text{D}_3$  and the  $1,25(\text{OH})_2\text{D}_3$  synthesized locally in T cells, monocytes, or dendritic cells, can affect T cell response either directly or indirectly, by modulating antigen presentation of dendritic cells to T cells [8]. Vitamin D is also a major regulator of T cell proliferation [87]. In immune-mediated diseases,  $1,25(\text{OH})_2\text{D}_3$  inhibited T cell proliferation, reduced inflammatory responses of Th1 and Th17 cells, while promoting the Th2 subtype, important for tissue repair [51]. This change is characterized by a switch in the cytokine profile. Interestingly, in infectious diseases where the Th1 and Th17 responses are necessary for the host response, this effect was not observed [88]. Regulatory T cell (Tregs)—lymphocytes that inhibit the immune response of other types of T cells—were induced by  $1,25(\text{OH})_2\text{D}_3$  in patients with renal disease, explaining the tolerogenic effect of vitamin D in renal transplant patients [89] (see also Chapter 74).

B cells play a crucial role in autoimmune diseases, such as rheumatoid arthritis, lupus erythematosus or type 1 diabetes, due to the production of autoantibodies. The modulatory effect of  $1,25(\text{OH})_2\text{D}_3$  on B cell activity is important in the autoimmune diseases mediated by B cells. In B cells,  $1,25(\text{OH})_2\text{D}_3$  suppressed immunoglobulin secretion and cell proliferation, and induced p27 expression and apoptosis, repressing naïve B cell differentiation and maturation to memory B and plasma cells [90]. High serum vitamin D levels were inversely associated with memory B cells, while vitamin D<sub>3</sub> restriction specifically promoted memory B cell development, accompanied by elevated levels of serum IgM, IgG1, IgG3, and anti-dsDNA IgG in a mouse model of systemic lupus [91].

Apart from their antigenic properties, to capture, process, and present antigens to adaptive immune cells

These activate different inflammatory cells, eventually resulting in an inflammatory microenvironment in tumors.  $1,25(\text{OH})_2\text{D}_3$  suppresses both the transcription factors and the production of chemokines, cytokines, and prostaglandins. It regulates activation and activity of inflammatory cells resulting in inhibition of cancer-related inflammation. *Solid arrows* indicate effect, *dotted lines* indicate inhibition. CD, cluster of differentiation; COX-2, cyclooxygenase 2; EMT, epithelial-to-mesenchymal transition; HIF-1, hypoxia-inducible factor 1; HLA-DR, human leukocyte antigen DR; IFN $\gamma$ , interferon gamma; Ig, immunoglobulins; IL, interleukin; NF- $\kappa$ B, nuclear factor kappa B; PD-L1, programmed cell death ligand 1; STAT3, signal transducer and activator of transcription 3; TGF $\beta$ , transforming growth factor beta; TLR, Toll-like receptor; TNF $\alpha$ , tumor necrosis factor alpha. Figure created with Biorender.com.





**FIGURE 87.2** Effect of  $1,25(\text{OH})_2\text{D}_3$  on receptors and mediators of inflammation-associated cancer.  $1,25(\text{OH})_2\text{D}_3$  inhibits the synthesis and activity of prostaglandins (PG), stimulating 15-PGDH expression leading to PG inactivation, suppressing COX-2 expression inhibiting the synthesis of PGs, and downregulating PG receptors.  $1,25(\text{OH})_2\text{D}_3$  inhibits STAT1-5 signaling, induces MKP5 expression that suppresses p38 activation resulting in decreased IL6 levels. By inhibiting NF- $\kappa$ B expression, either directly or by activating IGFBP3,  $1,25(\text{OH})_2\text{D}_3$  has a suppressive effect on proinflammatory cytokines, such as TNF $\alpha$ , IL12 $\beta$ , and IL8. Expression of TNF $\alpha$  is downregulated by suppressing TLR2 and TLR4.  $1,25(\text{OH})_2\text{D}_3$  inhibits HIF1 that leads to downregulation of VEGF and inhibition of the JNK and p38 pathways.  $1,25(\text{OH})_2\text{D}_3$  induces E cadherin expression and blocks  $\beta$  catenin nuclear activity, by inhibiting Wnt/ $\beta$  catenin signaling pathway. Arrows indicate activation, dotted lines indicate inhibition. CCL2, C-C motif chemokine ligand 2; HIF1, hypoxia-inducible factor 1; IFN- $\gamma$ , interferon gamma; IGFBP3, insulin-like growth factor-binding protein 3; I $\kappa$ B, inhibitor of NF- $\kappa$ B; IL, interleukin; JNK, c-Jun N-terminal kinase; MKP5, mitogen-activated protein kinase phosphatase 5; NF- $\kappa$ B, nuclear factor kappa B; PGE $_2$ , prostaglandin E $_2$ ; STAT1-5, signal transducer and activator of transcription 1–5; TLR, Toll-like receptor; TNF $\alpha$ , tumor necrosis factor alpha; VEGF, vascular endothelial growth factor. Figure created with Biorender.com.

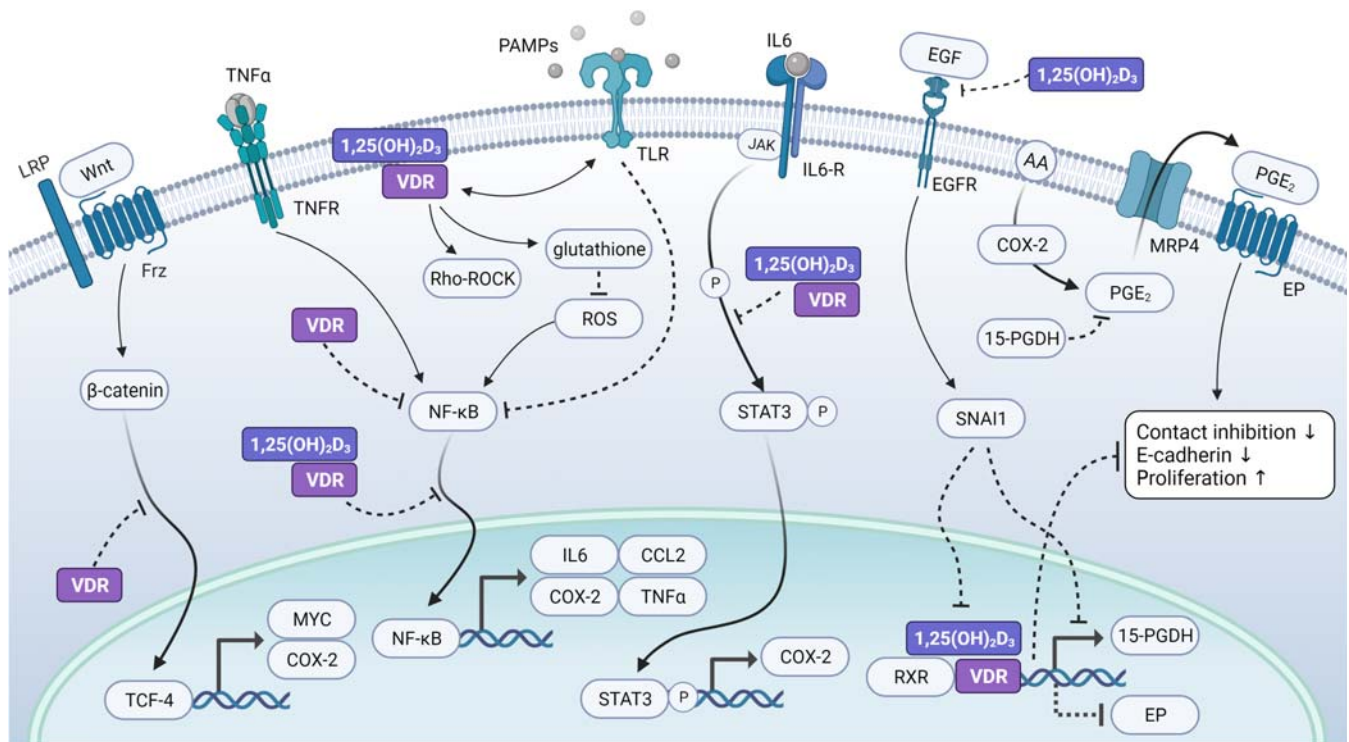
and mediate their polarization into effector cells, mature dendritic cells have the capacity to stimulate T cell activity. Exposure of mouse bone marrow–derived dendritic cells to  $1,25(\text{OH})_2\text{D}_3$  prevented their maturation and maintained a tolerogenic state that encouraged activation of the Treg phenotype.  $1,25(\text{OH})_2\text{D}_3$ -treated dendritic cells inhibited T cell proliferation and increased expression of the tolerogenic cytokines TGF $\beta$  and IL10 [92], and increased the expression of programmed death ligand 1 (PD-L1) [93]. Vitamin D signaling is essential also for T cell homing to the gastrointestinal tract. Defects of T cell homing might be one of the reasons for the increased inflammatory potential observed in the gastrointestinal tract of VDR knockout (VDR $^{-/-}$ ) mice. These animals express lower levels of the noncytotoxic CD8 $^{+}$  T cell variant CD8 $\alpha\alpha$  that seems to suppress gastrointestinal inflammation [94,95].

### 3.3 Effect of vitamin D in inflammatory diseases

Vitamin D deficiency is prevalent in several inflammatory and autoimmune diseases, such as type

1 diabetes, asthma, rheumatoid disorders, inflammatory bowel disease (IBD), lupus, and multiple sclerosis [96]. It has been suggested that the environmental factor that most strongly influences development of autoimmune diseases is vitamin D [97]. Genetic linkage analysis has revealed that rare inactivating mutations of *CYP27B1* seem to play a causative role in the development of multiple sclerosis [98]. Genome-wide association studies provided evidence that vitamin D deficiency probably plays a causal role in the pathogenesis of type 1 diabetes. Different inherited polymorphisms in the genes of the vitamin D system are associated with predisposition to type 1 diabetes [99]; e.g., low  $25(\text{OH})\text{D}_3$  serum levels due to the rs10741657G polymorphism of *CYP2R1* were associated with high incidence of type 1 diabetes [100,101]. A metaanalysis found that vitamin D reduced disease activity index in systemic lupus erythematosus patients [102].

Low vitamin D levels are prevalent also in IBD patients [103]. IBD, with its two forms ulcerative colitis (UC) and Crohn's disease (CD), is often a major risk factor of colorectal cancer (CRC). High doses of vitamin D



**FIGURE 87.3 Interactions of 1,25(OH)<sub>2</sub>D<sub>3</sub> with the signaling pathways associated with inflammatory bowel disease and colorectal cancer.** COX-2 expression is activated by inflammatory inducers (TNFα, PAMPs) through TNFR or TLR and activation of NF-κB or by mediators of proliferation (Wnt, EGF). Interaction between 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDR regulates proinflammatory TLR expression. Activated VDR suppresses NF-κB either directly or by increasing cytoplasmic glutathione levels that inhibit ROS expression. Inhibition of the NF-κB signaling pathway reduces the expression of proinflammatory mediators (TNFα, IL6, CCL2). Activated EGFR suppresses expression of 15-PGDH, the enzyme catabolizing PGE<sub>2</sub>, by inducing SNAI1. The intracellular production of PGE<sub>2</sub> from AA by COX-2 is highly stimulated in inflammatory bowel disease. PGE<sub>2</sub> is transported out of the cell by MRP4, while into the cell by PGT. PGE<sub>2</sub> binds the prostaglandin EP and stimulates signaling. 1,25(OH)<sub>2</sub>D<sub>3</sub>-bound VDR blocks PGE<sub>2</sub> accumulation by inhibition of EP2 and induction of 15-PGDH expression. 1,25(OH)<sub>2</sub>D<sub>3</sub> also suppresses EGFR expression. Solid arrows indicate activation, and dotted lines indicate inhibition. Gradient arrows indicate translocation. 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; COX-2, cyclooxygenase 2; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EP, prostaglandin E receptor; IL6, interleukin 6; CCL2, C–C motif chemokine ligand 2; MRP4, multidrug resistance–associated protein 4; NF-κB, nuclear factor kappa B; PAMP, pathogen-associated molecular pattern; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PGT, prostaglandin transporter; ROS, reactive oxygen species; SNAI1, SNAIL drosophila homolog of 1; TLR, Toll-like receptor; TNFα, tumor necrosis factor alpha; TNFR, tumor necrosis factor receptor; VDR, vitamin D receptor. Figure created with Biorender.com.

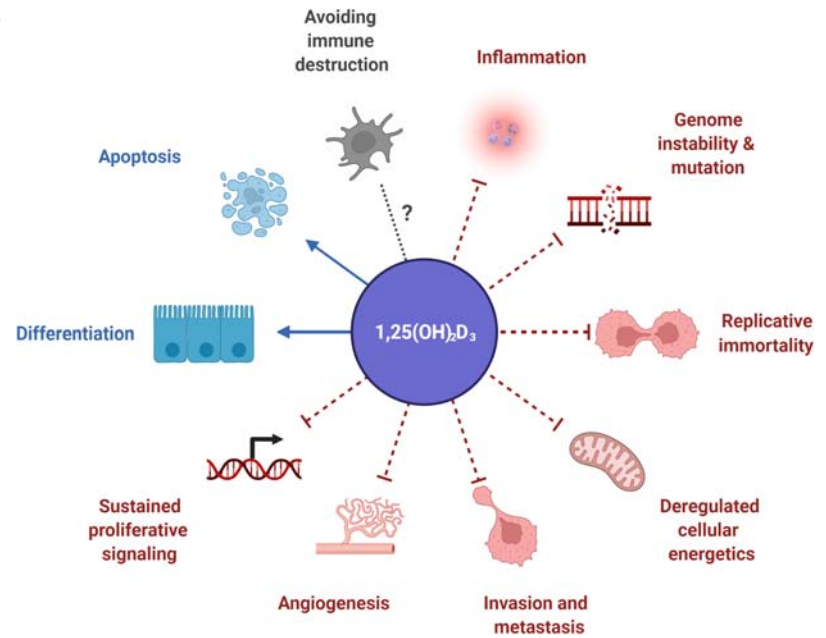
or normalization of serum vitamin D levels led to remission and reduced risk for surgery in patients with mild to moderate Crohn's disease [104–107]. In animal models of IBD, vitamin D prevented or ameliorated inflammation and clinical disease [108–110].

To summarize, inflammation is caused by a variety of factors and is associated with a wide range of diseases, including cancers and autoimmune disorders. VDR and the vitamin D metabolizing enzymes are expressed almost ubiquitously in the innate and adaptive immune system. The interaction between vitamin D and inflammation is complex. On the one hand, vitamin D inhibits inflammation; on the other hand, inflammation affects vitamin D metabolism and signaling. The potential of vitamin D to inhibit inflammation depends on the disease characteristics and on the levels of the vitamin D metabolites.

#### 4. Inflammation-associated cancer

Inflammation and “evading immune destruction” were eventually added to the hallmarks of cancer [1]. The hallmarks of inflammation-related cancer include the presence of inflammatory cells and inflammatory mediators in the tumor, activated tissue remodeling and angiogenesis, similar to that seen in chronic inflammatory responses, and tissue repair [42]. The active vitamin D metabolite 1,25(OH)<sub>2</sub>D<sub>3</sub> affects most cancer hallmarks. It inhibits proliferation, angiogenesis, invasion, and metastasis [13], alters cellular metabolism, induces differentiation and apoptosis, and inhibits protumorigenic inflammation [9,111] (Fig. 87.4). Main drivers of cancer are highly active oncogenes in parallel with silenced tumor suppressors. Many of the oncogenes upregulated in several cancers, such as growth

**FIGURE 87.4** Effect of  $1,25(\text{OH})_2\text{D}_3$  on the hallmarks of cancer. Figure created with Biorender.com.



factors, growth factor receptors, kinases, and transcription factors, are also involved in the inflammatory process.

The normal inflammatory mechanisms, essential for tissue regeneration and the fight against infections, resolve after healing, but chronic inflammation can promote tumorigenesis, due to a feed-forward loop of inflammatory cell recruitment and inflammation-induced signaling. Around 15%–20% of all cancers are preceded by chronic inflammation or infection; e.g., IBD, chronic hepatitis, or pancreatitis, leading to CRC, liver, or pancreatic cancer, respectively [2]. The immune system, as part of the tumor microenvironment, can act both antitumorigenic, enabling immune-surveillance, and protumorigenic, blocking antitumor immunity and secreting tumor-promoting signals [2]. “Hot”, or immunogenic tumors, show signs of inflammation and are usually responsive to cancer immunotherapy. In inflammation-associated cancer, numerous pathways are active that suppress antitumor immunity, supporting cancer-promoting inflammation. A central question is, how to switch the tumor-promoting immune-suppressive microenvironment to a tumor-destroying state [2].

The role of vitamin D in modulating the immune surroundings in cancer is unclear. Ample evidence suggests that it promotes antitumorigenic phenotype of cancer cells and the cancer-associated microenvironment, but it also induces expression of PD-L1 the ligand of the immune-checkpoint inhibitor (programmed cell death protein 1), which could lead to evasion from immune-surveillance [112]. In a recent study, however, high

serum PD-L1 levels correlated significantly with low levels of vitamin D [113]. There are no clear-cut studies suggesting that vitamin D enables evasion from immune-surveillance. There is some evidence [114] that similarly to the Th1 and Th17 response regulation in infectious diseases [88], in tumors, vitamin D supports immune surveillance [114]. Morita et al. found that in patients with digestive tract cancers, vitamin D supplementation upregulated PD-L1 levels only in patients with lowest PD-L1 levels (quintile Q1), but reduced PD-L1 expression in patients with the highest quintile (Q5). In this group of patients (Q5), vitamin D supplementation reduced also the risk of relapse and mortality [114]. The vitamin D target gene IL10 is able, on the one hand, to stimulate antitumor immunity and, on the other, to inhibit tumor-associated inflammation [115]. Additionally, vitamin D inhibits epithelial-to-mesenchymal transition (EMT) [116], a process often supported by inflammatory markers and needed for invasion [117].

In the following sections, we will focus on the effect of vitamin D on the pathways that link inflammation and tumorigenesis.

#### 4.1 Key factors in inflammation-associated cancer

The interplay between inflammation and cancer is complex and affects the progression of a wide variety of solid tumors [118,119]. Inflammation and cancer are linked by two major pathways: an extrinsic pathway, driven by infections or inflammatory conditions that



enhance cancer risk and an intrinsic pathway, driven by genetic alterations that cause both transformation and inflammation [42]. Extrinsic circumstances, such as chronic inflammation in several tissues (colon, prostate, pancreas, liver), create conditions that favor promotion and progression of cancer. The intrinsic pathway links oncogene activation and/or tumor suppressor silencing with local inflammation. In transformed cells, inflammatory responses sustain survival and proliferation, leading to tumor promotion [120]. It has been hypothesized that oncogenes, regardless of their molecular class, coordinate inflammatory transcriptional programs that are linked to angiogenesis and to recruitment of cells of the myelomonocytic lineage [42]. Usually, both the intrinsic and the extrinsic pathways are needed to promote tumor formation (Fig. 87.1).

Among the key intrinsic factors triggering inflammation-associated cancer are transcription factors such as NF- $\kappa$ B and STAT3, as well as inflammatory cytokines (e.g., IL1 $\beta$ , IL6, IL17, IL23, TNF $\alpha$ , IFN- $\gamma$ ), chemokines (e.g., IL8, CCL2, CCL5), prostaglandins (PGE<sub>2</sub>, PGF<sub>2 $\alpha$</sub> ), survival molecules (survivin, Bcl-2, Bcl-xl), and molecules linked to metastasis (MMPs, VEGF) [22,119,121].

#### 4.1.1 Nuclear factor $\kappa$ B pathway

NF- $\kappa$ B, the key coordinator of inflammation, is also considered a major endogenous tumor promoter [122]. It has several subunits, the classical p50, p65, and the nonclassical p52 and Rel-B [123]. I $\kappa$ B, the inhibitor of NF- $\kappa$ B, sequesters the subunits p50 and p65 in the cytoplasm. I $\kappa$ B is degraded upon phosphorylation by IKK $\beta$  and subsequent ubiquitination, enabling translocation of p50 and p65 into the nucleus, where NF- $\kappa$ B induces transcription of both pro- and antiinflammatory cytokines (e.g., IL1, IL4, IL6, IL10, IL12, IL17, IL23), chemokines (e.g., IL8, CCL2, CXCL12), antiapoptotic molecules (Bcl-2, Bcl-xl, survivin), matrix metalloproteinases (MMPs), and adhesion molecules (VEGF, TWIST, CXCR4) [124]. In tumors, NF- $\kappa$ B activation is mainly driven by inflammatory cytokines within the tumor microenvironment [125] and leads to transcription of inflammatory cytokines, COX-2, inducible nitric oxide synthase (iNOS), adhesion molecules, angiogenic factors, and antiapoptotic genes [44]. NF- $\kappa$ B is important also in determining the balance between the tumor promoting and antitumor properties of macrophages.

#### 4.1.2 Signal transducers and activators of transcription 3 pathway

STAT3 is a pleiotropic transcription factor, activated in both immune and tumor cells. It is activated through phosphorylation by numerous cytokine and growth factor receptors. STAT3 signaling is one of the major intrinsic inflammatory pathways involved also in

tumorigenesis [126,127]. In inflammation-driven cancers, STAT3 is constitutively activated by high levels of the proinflammatory cytokine IL6 in a feed-forward loop with itself and NF- $\kappa$ B [126,128]. It increases the capacity of tumors to evade the immune system, induces oncogenesis and cancer cell stemness, and inhibits apoptosis [119,129–131].

#### 4.1.3 Prostaglandin pathway

Activation of PG signaling is an early event during progression from inflammation to malignancy [19,54,132]. PGs are bioactive lipids derived from arachidonic acid. The rate-limiting enzyme in PG synthesis is the PG-endoperoxide synthase, also known as cyclooxygenase (COX). COX-1 is constitutively expressed, while expression of the inducible isoform COX-2 is rapidly induced by growth factors, cytokines, and tumor promoters and is often overexpressed in tumors [133]. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is the enzyme responsible for the catabolism of PGs [134]. COX-2 is expressed both in epithelial cells and in infiltrating macrophages, and COX-2/PGE<sub>2</sub> signaling is essential in promoting tumor progression and encouraging metastatic spread [132,135]. COX-2 is upregulated in inflamed tissues such as the mucosa of IBD patients and in colorectal tumors, while 15-PGDH expression is reduced [136–138]. High COX-2 levels were seen in prostate, in areas of proliferative inflammatory atrophy (PIA) [139]. In prostate tumors, COX-2 expression is a prognostic factor for high-risk disease [140,141]. In breast cancer, COX-2 levels were high in aggressive tumors [142]. Chemotherapy often induces COX-2/PGE<sub>2</sub> signaling, which then impairs the efficacy of immunotherapies [143]. Synthesis of PGs at sites of infiltration of inflammatory cells in tumors contributes to tumor progression by stimulating expression of genes involved in proliferation.

#### 4.1.4 Wnt/ $\beta$ -catenin pathway

Canonical Wnt signaling is mediated by a complex between the frizzled receptor (FZD) and its coreceptor, the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6). In the absence of Wnt activators,  $\beta$ -catenin is bound and phosphorylated in a complex of proteins involving the scaffold protein axin2, adenomatous polyposis coli (APC), and the serine-threonine kinases CK-1 and GSK-3 $\beta$  for subsequent proteasomal degradation. Activation through Wnt ligands leads to nuclear translocation of  $\beta$ -catenin, association with the T cell factor/lymphoid enhancer factor (Tcf/Lef) transcription factors, and transcription of genes regulating among others proliferation, differentiation, tissue regeneration, and wound healing [144,145]. Aberrant activation of the Wnt/ $\beta$ -catenin pathway is one of



the first steps toward malignant transformation in sporadic CRC.

In numerous inflammation-associated cancers, there is a cross-talk between the Wnt/ $\beta$ -catenin pathway and the inflammatory cascade [47,48,146], partially through the NF- $\kappa$ B pathway [147,148]. Activation of Wnt/ $\beta$ -catenin signaling induces COX-2 in cancer cells. The resulting increase in PGE<sub>2</sub> triggers aberrant Wnt/ $\beta$ -catenin pathway and promotes inflammatory cancer progression, also by upregulating IL1 $\beta$  and IL8 secretion from macrophages [149]. The immune cells in the microenvironment of the tumors play an essential role in tumor development and metastasis. Tumor-associated fibroblasts (TAFs) are important sources of tumor-promoting chemokines, cytokines, and growth factors. Chemokines, their receptors, and cytokines coordinate the autocrine and paracrine interactions between neoplastic cells and TAFs. Coculture of tumor cells with macrophages increased the invasive capacity of tumor cells in an NF- $\kappa$ B- and TNF $\alpha$ -dependent manner, affecting their ability to colonize the metastatic niche [150].

## 4.2 Vitamin D and inflammation-associated cancer

Numerous epidemiological studies suggest that high levels of serum 25(OH)D<sub>3</sub> prevented the development of different cancers and increased the survival rate of patients [151–156]. Interventional studies, however, often failed to show such an effect for increased vitamin D<sub>3</sub> intake [157–159]. The reasons for the contradictory results of the interventional studies could be manifold, including individual differences in responsiveness to vitamin D<sub>3</sub>, insufficient dosage, inadequate duration of the intervention, and poor compliance. The antitumorogenic actions of vitamin D<sub>3</sub> were more convincing in animal studies [160,161], possibly due to the strictly controllable experimental conditions (“lifestyle,” diet) and the genetic homogeneity of the animals.

The strongest evidence on the protective role of high serum 25(OH)D<sub>3</sub> levels against different types of cancer was obtained in association with colon, prostate, and breast cancers (see more in Chapter 84).

### 4.2.1 Molecular pathways regulated by vitamin D in inflammation-associated cancer

Vitamin D prevents inflammation-associated cancers by regulating events downstream both the extrinsic and intrinsic pathways (Fig. 87.1). It ameliorates severity of chronic inflammations by regulating the innate and adaptive immune system [51]. Additionally, VDR-bound 1,25(OH)<sub>2</sub>D<sub>3</sub> modulates transcriptional activity of genes involved in proliferation, differentiation,

apoptosis, migration, and inflammation in an autocrine or paracrine way and regulates a complex network that controls both normal and malignant cell growth [9,10,13]. Both in inflammation and in many tumors, expression of the VDR, the synthesis, and the catabolism of vitamin D metabolites are altered, compromising 1,25(OH)<sub>2</sub>D<sub>3</sub> sensitivity and signaling [9]. The genes regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> in tumors are only partially the same as the genes regulated in the corresponding normal tissue [162].

Vitamin D regulates all the major pathways driving inflammation-associated cancer, modulating both cancer-related and inflammation-related signaling (Figs. 87.1 and 87.3) [163]. Gene ontology analysis showed that in the leukemia cell line THP-1, the most significant processes regulated by the treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> were immune functions, such as neutrophil signaling and inflammatory responses [164]. The intrinsic pathway driven by the c-MYC oncogene was targeted by the growth inhibitory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> [165]. Vitamin D prevented tumor angiogenesis by reducing expression of several angiogenic factors (VEGF, HIF-1, IL8). It suppressed PG synthesis and signaling and induced PG degradation. The dual phosphatase MKP5 dephosphorylates and thus inactivates several mitogen-activated kinases. Dephosphorylation of p38 leads to inhibition of proinflammatory cytokine (e.g., IL6) production. 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced MKP5 expression, leading to subsequent inhibition of the p38 signaling, inhibited invasion, and metastasis by preventing MMP9 upregulation. 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment modulated the RHO-ROCK pathway and increased E-cadherin expression (see for review: [13,19,166]).

### 4.2.2 Effect of vitamin D in inflammation-associated colorectal cancer

Inflammation is a major risk factor for CRC. Patients with inflammatory bowel diseases have a higher risk to develop gastrointestinal cancers depending on the type of IBD, location, and extent of the disease [167,168]. Diet and lifestyle contribute to the progression from IBD to cancer either directly, by affecting expression or activity of genes interacting with the inflammatory pathways, or indirectly, through their effect on the immune system and the microbiota [169–172].

Numerous molecular alterations responsible for development of sporadic CRC are also involved in colitis-associated colonic carcinogenesis, although some of the pathways are different, or are altered differently [170]. Comparing the genomic landscape of IBD-associated CRC (colitis-associated CRC, CAC) with that of sporadic colorectal tumors, Robles et al. [173] found similar mutations in the pathways triggered by PI3K, BRAF, ERBB2, and TGF $\beta$ . Some of the key mutations in sporadic colorectal tumors, e.g., KRAS

mutations were not common in CAC, suggesting differences in the etiology of IBD-associated CRC and that of sporadic CRC. In over 80% of sporadic colorectal tumors, the Wnt/ $\beta$ -catenin pathway is altered, mainly due to inactivating mutations of adenomatous polyposis coli (*APC*). In contrast, genes of the Wnt/ $\beta$ -catenin pathway were mutated only in about 53% of the CACs. However, most of the mutations were in the gene coding for the *SOX9* transcription factor and not in *APC* [173]. Another cause of the Wnt/ $\beta$ -catenin activation in CAC was the methylation-dependent silencing of Wnt inhibitors [174]. A further study found that mutations in *TP53*, *IDH1*, and *c-MYC* were much more frequent in CAC than in sporadic CRC [175]. Genetic alterations in genes associated with cell-to-cell communication were more prevalent in the IBD-associated tumors; furthermore, the Rho-Rac pathway was mutated in 50% of CAC tumors [173].

Vitamin D serves also as an immunoregulatory micro-nutrient, necessary for maintaining gut epithelial integrity, prevention of IBD, and inflammation-associated CRC (Fig. 87.3) [106,176,177]. IBD patients are very often vitamin D deficient [107], and the vitamin D system is often deregulated in the intestine of both IBD and in CRC patients. *VDR* expression is significantly lower in the intestines of IBD patients [178]. In patients with active CD, *VDR* expression levels are decreased in comparison with patients with quiescent disease and correlate inversely with inflammation [179,180]. *CYP27B1* protein expression is low in normal colonic mucosa. In chronically inflamed tissues, *CYP27B1* is upregulated as a defense mechanism to enhance tissue concentration of 1,25(OH) $_2$ D $_3$ . The 1260 TT polymorphism in *CYP27B1* gene was associated with higher calprotectin levels [181]. Adenomas and moderately differentiated adenocarcinomas also express high levels of *CYP27B1*, whereas in undifferentiated areas of high-grade tumors, *CYP27B1* is undetectable [182]. An active and functioning *CYP27B1*/*VDR* system in the colon is needed to prevent cancer-initiating COX-2 activity [183]. *VDR* expression is lost also in undifferentiated colorectal tumors, while the catabolizing *CYP24A1* is overexpressed [184–186]. In a xenograft model, overexpression of *CYP24A1* led to more aggressive tumors [187]. In African Americans, two SNPs (single-nucleotide polymorphisms) of *CYP24A1* correlated positively with CRC risk [188].

There is a significant influence of geographical latitude on the incidence of IBD [189,190] and CRC [191], with higher incidence in the north than in the south. Evidence from the majority of epidemiologic studies confirm a positive association between the concentration of serum 25(OH)D $_3$  and survival rate [192] and a negative association with the risk of colorectal neoplasms in patients with IBD, or sporadic CRC [193–195]. One

mechanism by which vitamin D affects IBD severity and prevents development of CAC is by regulating gut microbiota–host interactions through the synthesis of antimicrobial peptides [196].

In a randomized placebo-controlled trial, daily intake of 800 IU vitamin D $_3$  reduced serum levels of different inflammation markers (e.g., CRP, TNF $\alpha$ , IL6, IL8, IL1 $\beta$ , IL10), decreased the inflammation score by 77%, and was associated with lower risk of CRC [197]. Daily supplementation of healthy volunteers receiving the fat-rich Western-style diet with 0.5  $\mu$ g 1,25(OH) $_2$ D $_3$  upregulated in the rectal mucosa the expression of numerous genes involved in inflammation and immunity [198]. A systematic review found that high doses of vitamin D improved clinical and biochemical disease activity scores in vitamin D–deficient IBD patients [199]. In contrast, vitamin D $_3$  supplementation was not able to reduce the risk of recurrence in patients with resected colorectal adenomas, not even in patients with advanced adenocarcinoma having low serum 25(OH)D $_3$  levels [200].

The effect of vitamin D in animal models is much more consistent. Mice lacking either *Vdr* or *Cyp27b1* are highly susceptible to colitis and colitis-associated cancer [201]. In *Vdr* $^{-/-}$  mice, the tight junctions of intestinal epithelial cells are disrupted, wound healing mechanisms are impaired, and the intestinal transepithelial electric resistance is very low [202,203]. *Vdr* loss significantly increased tumorigenesis and activated EGFR and  $\beta$ -catenin signaling in chemically induced CAC [204]. The gut microbiome is changed in mice lacking *Vdr* in their intestine [205]. Reintroduction of *VDR* in the intestinal epithelial cells of *Vdr* $^{-/-}$  mice rescued them from colitis and death [206]. A study on *Cyp27b1* knockout mice demonstrated that 1,25(OH) $_2$ D $_3$  deficiency triggered growth and multiplicity of colon tumors, and generation of inflammatory cytokines that caused cellular senescence and DNA damage [207].

In experimental models, supplementation of vitamin D protected against inflammation and inflammation-associated cancer [156,208]. Vitamin D supplementation reduced dysplasia score in a dose-dependent manner in the azoxymethane (AOM)/dextran sulfate sodium (DSS)–induced colonic tumorigenesis model [209]. In a similar model, different vitamin D analogs reduced the number of colitis-associated tumors after 16 weeks [210].

#### 4.2.3 Vitamin D–dependent gene expression in inflammatory and cancer-promoting pathways in the intestines

In in vitro experiments, 1,25(OH) $_2$ D $_3$  inhibited most of the pathways (Wnt/ $\beta$ -catenin, NF- $\kappa$ B, PG pathway) responsible for the development of inflammation-associated cancer (Fig. 87.3) [10,211]. Treatment of established CRC cell lines with 1,25(OH) $_2$ D $_3$  inhibited the

expression of genes involved in proliferation and migration [212,213] and suppressed the Warburg effect [214].  $1,25(\text{OH})_2\text{D}_3$  also reversed cribriform morphology and adjusted spindle orientation—morphological characteristics of human colon cancer cells—by upregulating the expression of the tumor suppressor PTEN and stimulating CDC42 and PKC $\zeta$  [215].  $1,25(\text{OH})_2\text{D}_3$  induced expression of E-cadherin, GADD45, and EFTUD1 in both CRC cell lines and patient-derived colonic organoids [213]. It antagonized the Wnt/ $\beta$ -catenin pathway on multiple levels.  $1,25(\text{OH})_2\text{D}_3$  treatment prevented TCF/ $\beta$ -catenin-dependent transcription by binding of VDR to  $\beta$ -catenin, sequestered  $\beta$ -catenin to the tight junctions by supporting E-cadherin binding to  $\beta$ -catenin, and induced expression of the Wnt inhibitor DICKKOPF-1 [211].  $1,25(\text{OH})_2\text{D}_3$  blocked TGF $\beta$ 1/ $\beta$ 2-induced migration and invasion, upregulated E-cadherin expression, and inhibited the expression of N-cadherin, preventing the so called “cadherin switch” that plays an essential role in tumor invasiveness. Additionally, the level of MMP-2 and MMP-9 was decreased, inhibiting the invasion of the cancer cells into the circulatory system [216].  $1,25(\text{OH})_2\text{D}_3$  also prevented EMT, which is involved in the metastatic process [116,217].

In a bacteria-driven colitis model, high-dietary vitamin D—suppressed NF- $\kappa$ B activity and MAPK signaling in colonic epithelial cells. This led to reduced inflammatory cell infiltration and expression of proinflammatory cytokines in the cecum, and fewer invasive tumors in Smad3 $^{-/-}$  mice infected with *Helicobacter bilis* [108]. Vitamin D supplementation reduced tumor burden in the AOM/DSS model of CAC by inhibiting the transcriptional activity of NF- $\kappa$ B and p-38 MAPK [218]. The vitamin D analog Ro26-2198 significantly decreased expression of Cox-2, c-Myc, and pERK, thus delayed the onset of clinical colitis, and inhibited the development of dysplastic foci in an AOM/DSS model of CAC [219]. Vitamin D deficiency increased disease severity and mortality in the AOM/DSS colitis-associated cancer model [220]. Conversely, AOM/DSS treatment reduced serum  $25(\text{OH})\text{D}_3$  levels and intestinal VDR expression, at the same time increasing COX-2 levels. Challenging these mice with a vitamin D—deficient diet worsened the colitis score and increased mortality [221].

A further protective action of vitamin D against CAC is improving bacteria-induced impairment of the intestinal barrier function [222] and the upregulation of molecules important for maintaining intestinal integrity, such as claudins [109,208]. The intestinal vitamin D system supports mucosal barrier integrity by maintaining a healthy balance between proliferation and apoptosis of the epithelial cells [223]. An important factor that affects the antiinflammatory and antimitogenic effects of vitamin D in the colon is calcium intake [224,225].

Calcium supplementation and vitamin D status appear to act largely together, in an interdependent way [226,227]. Vitamin D supplementation for a year reduced COX-2/15-PGDH ratio in the normal rectal mucosa of patients with colorectal adenomas. The effect was even higher when vitamin D was given together with calcium [228].

Considering all these effects, vitamin D supplementation or less calcemic vitamin D analogs could be useful in ameliorating IBD and preventing inflammation-associated colorectal cancer. For additional information on the effect of vitamin D in IBD and CRC, see also Chapters 88 and 97.

#### 4.2.4 Effect of vitamin D in inflammation-associated prostate cancer

Prostate cancer (PCa) is the fourth most prevalent cancer in men worldwide, with an increasing incidence rate in most countries [229–232]. Inflammation is crucial in the etiology and development of PCa [233]. Chronic inflammation in noncancerous prostate correlated positively with development of PCa [234]. Areas of proliferative inflammatory atrophy (PIA) in the prostate are precursors of intraepithelial neoplasias (PIN) and carcinomas [139]. The source of inflammation is usually unknown. Exposure to infections, hormones, or dietary carcinogens could damage the prostate, leading to chronic inflammation and PIA. Even the normal prostate gland contains inflammatory cells, such as T and B lymphocytes, macrophages, and mast cells [139]. Both stromal and epithelial cells express immune-modulatory genes and contribute to the inflammatory environment. The stroma becomes reactive early in the PCa development and affects epithelial cell proliferation, differentiation, and apoptosis and mediates the immune response of epithelial cells during cancer progression [235,236]. Proinflammatory cytokines, released by T lymphocytes and macrophages, induce COX-2 expression in adjacent tumor cells and stimulate angiogenesis in stromal tissues [237]. Interestingly, however, a prospective cohort study found that the presence of chronic inflammation in the prostate tumor was associated with better prognosis for the patients [238].

The vitamin D system is often deregulated in PCa, impairing the cancer preventive action of vitamin D [31]. High expression of VDR in the tumor tissue was associated with reduced risk of lethal PCa [239]. CYP27B1 expression is reduced in prostate tumors [240] due to promoter hypermethylation [241], or repression by the transcription factor growth factor independence-1 [242]. Higher levels of vitamin D metabolizing enzymes were detected both in the benign and cancerous prostate epithelia compared with the surrounding stroma [243]. Two VDR SNPs were associated with prostate cancer risk, independent of the age



of the participant or the aggressiveness of the tumor [244].

The epidemiologic evidence regarding the antiinflammatory and cancer preventive effect of vitamin D in prostate diseases is mixed. Low 25(OH) $D_3$  serum levels were associated with inflammation, increased odds of adverse pathology, aggressive tumors, and higher mortality [32,245–247]. Association with overall risk is less convincing [248,249]. There are even studies that found a U-shaped effect [250]. In Finland, the highest risk for developing prostate cancer was for men in the highest tertile of 25(OH) $D_3$  level, whereas mortality was highest in the lowest 25(OH) $D_3$  tertile [251]. Other studies found that high serum levels of 25(OH) $D_3$  correlated with better survival in all stages and grades of PCa [30,252] and that posttreatment 1,25(OH) $_2D_3$  levels in patients with aggressive tumors inversely correlated with all-cause and PCa-specific mortality [253]. More-and-more evidence is pointing toward racial disparities in the incidence and mortality from PCa [254–256]. Whether the fact that African Americans have a higher incidence rate of PCa than Caucasians is due mainly to socioeconomic factors, or also due to the lower vitamin D levels prevalent in this population, is not yet clear [257].

Vitamin D supplementation reduced inflammation and tumor size in PCa patients by upregulating growth differentiation factor-15, an antiinflammatory molecule markedly reduced in PCa [258]. In a pilot study, supplementation of 2,000 IU of vitamin D per day for 21 months increased prostate-specific antigen (PSA) doubling time in PCa patients [259]. Combining 1,25(OH) $_2D_3$  with the nonsteroidal antiinflammatory drug naproxen prolonged PSA doubling time compared with baseline in more than 75% of patients with early recurrent PCa [260].

Several mouse models have demonstrated the cancer preventive effect of vitamin D. A high vitamin D diet (5,000 IU/kg) or 1,25(OH) $_2D_3$  administration significantly inhibited growth of PC3 cell xenografts in mice [261]. In the TgAPT $_{121}$  mouse model for early steps of prostate carcinogenesis, disruption of the vitamin D signaling accelerated the development of tumors from high-grade PIN, while high-dietary vitamin D intake reduced tumor incidence [262]. A diet low in vitamin D (25 IU VD/kg diet), or *Vdr* deletion, supported procarcinogenic transformation that accelerated PCa development [263]. In a mouse model of PCa that recapitulates the various steps of tumor development from the prostatic intraepithelial neoplastic lesions to adenocarcinoma, 1,25(OH) $_2D_3$  inhibited progression from low-grade PIN to high-grade PIN if administered before neoplastic transformation [264]. In the TRAMP model of PCa, administration of vitamin D during early stages initially prevented the development of aggressive PCa, but a prolonged administration seemed to increase the

aggressiveness of the metastatic process [265]. Lack of *Vdr* accelerated tumor progression in the LPB-Tag model of PCa as well [266]. The expression of the vitamin D metabolizing enzymes in the prostate of rats changes during aging: *Cyp27b1* expression decreased with age, mainly in areas with hyperplasia, premalignant lesions, and tumors, while expression of *Cyp24a1* increased in both the normal and transformed prostate [267].

#### **4.2.5 Vitamin D–dependent gene expression in inflammatory and cancer-promoting pathways in the prostate**

Vitamin D regulates not only the epithelial cells but also different other cell types, such as immune cells or stromal cells, and modulates the complex interactions among them. In prostate epithelial cells, 1,25(OH) $_2D_3$  treatment modulated a complex network of pathways that would slow or restrain tumorigenesis. An integrative network-based analysis of publicly available mRNA and microRNA datasets showed that in the LNCaP prostate cancer cells, 1,25(OH) $_2D_3$  treatment affected 15 signaling pathways, mostly inhibiting cell cycle–related and cancer-specific processes [268]. In the normal prostate cell line RWPE1, 1,25(OH) $_2D_3$  treatment inhibited pathways regulating proliferation, angiogenesis, and induced proapoptotic ones. The major inflammatory pathways, COX-2/PGE $_2$ , and NF- $\kappa$ B, are also active in PCa and often correlate with lower vitamin D levels [32]. 1,25(OH) $_2D_3$  suppressed Wnt/ $\beta$ -catenin, NF- $\kappa$ B, Notch signaling, inhibited the JAK-STAT3 inflammatory pathway reducing the expression of the proinflammatory cytokines IL1, IL17, IL8, and IL6 [269], and suppressed early prostate carcinogenesis [262]. In normal human prostate cells and in primary tumor-derived cancer cells, 1,25(OH) $_2D_3$  induced MKP5 expression in a VDR-dependent manner and suppressed TNF $\alpha$  signaling. This led to inhibition of p38 activity and IL6 synthesis, blocking the procarcinogenic inflammatory processes [270]. 1,25(OH) $_2D_3$  inhibited NF- $\kappa$ B signaling by preventing nuclear translocation of the NF- $\kappa$ B subunit p65. The Rel-B subunit is overexpressed in androgen-independent tumors with high Gleason scores, conferring radiation resistance. 1,25(OH) $_2D_3$  suppressed radiation-mediated activation of the Rel-B subunit, enhancing sensitivity of androgen-dependent PCa cells to ionizing radiation [271].

Several studies suggest a central role for COX-2 in prostate tumorigenesis [54]. It was even suggested that COX-2 expression is an independent predictor for PCa recurrence [272]. 1,25(OH) $_2D_3$  inhibited the COX-2/PGE $_2$  pathway both in established PCa cell lines and in primary PCa cells. It reduced expression of COX-2 and of PG receptors, and induced 15-PGDH levels,



inhibiting PGE<sub>2</sub>-mediated proliferative, proangiogenic, and antiapoptotic signaling [54,273]. Another way by which VDR-bound 1,25(OH)<sub>2</sub>D regulates antiinflammatory and antitumorigenic pathways in the prostate is by regulating the expression of DICER1 and several microRNAs, such as miR-126-3p, miR 154-5p, and miR-21-5p [274].

Dietary supplementation with 1,25(OH)<sub>2</sub>D<sub>3</sub> and soy inhibited the growth of PCa xenografts in a mouse model. It increased expression of antiproliferative (p21, IGFBP-3) and proapoptotic (Bax) genes, inhibited antiapoptotic (Bcl-2) genes, and suppressed the PG pathway (COX-2, 15-PGDH, PG receptors) [275]. Vitamin D supplementation was able to prevent high-calcium diet-induced progression of PIN, by inhibiting the upregulation of the expression of the cationic channel TRPC6, and the calcium-sensing receptor (CaSR) [276].

Understanding the mechanism of action of vitamin D in PCa could lead to new strategies in using vitamin D for the benefit of PCa patients. Both preclinical and clinical studies showed that vitamin D potentiates the action of different drugs that have antitumor effect in PCa [277,278]. For additional information, see also Chapter 91.

#### **4.2.6 Effect of vitamin D in inflammation-associated breast cancer**

Breast cancer (BCa) is one of the most frequent cancers and the main cause of cancer-related deaths in women [279]. It is a very heterogeneous disease. The etiology is not completely understood; therefore, it is not clear how much impact has inflammation in breast tumorigenesis [280]. Evidence is mounting though that innate and adaptive leukocytes play an important role in breast carcinogenesis, as human BCa development is paralleled by abundant infiltration of immune cells [281]. Activated B cells were found in tumor-associated stroma, mainly in early breast tumors, while infiltrating T lymphocytes are more abundant in higher-grade ductal carcinomas in situ (DCIS) and in invasive carcinomas. The percentage of regulatory T cells in the tumor and surrounding stroma increases in parallel with disease stage and promotes immunosuppression and tumor progression [281].

The COX-2/PGE<sub>2</sub> pathway seems to promote Treg differentiation [282]. An active IL6/JAK/STAT3 signaling is important for the invasiveness and metastatic potential of breast tumor cells [283]. The cytokine IL1 plays a central role in the intercellular and intracellular cross-talk in the microenvironment of breast tumors [284]. Vitamin D is able to inhibit all these pathways. Breast epithelial cells express the vitamin D system and have the capacity to synthesize the active vitamin

D metabolite 1,25(OH)<sub>2</sub>D<sub>3</sub>. Vitamin D signaling and metabolism becomes deregulated during BCa progression [285]. Both VDR expression and 1,25(OH)<sub>2</sub>D<sub>3</sub> action alter with the transformation process [111]. The VDR is expressed in normal breast tissue; in benign and malignant tumors, however, its levels decrease with invasiveness of the tumor. VDR levels correlated with estrogen receptor (ER) $\alpha$  in breast carcinomas. CYP27B1 expression decreased during malignant progression, while CYP24A1 expression increased [286].

The findings of epidemiological studies regarding the effect of vitamin D intake and 25(OH)D<sub>3</sub> serum levels on risk, progression, aggressiveness, and survival/mortality of BCa are mixed. Randomized controlled trials observed only slight benefits of vitamin D supplementation on BCa development [111,287]. A metaanalysis found that concentrations higher than 50 ng/mL 25(OH)D<sub>3</sub> in the serum were associated with 50% lower risk of developing BCa [288,289], while other studies found no inverse association [290]. A randomized placebo-controlled trial detected no correlation between low (400 IU) daily doses of vitamin D<sub>3</sub> and BCa risk in postmenopausal women [291,292]. Higher vitamin D<sub>3</sub> intake (1100 IU/day), which raised 25(OH)D<sub>3</sub> serum concentrations up to more than 80 nmol/L, had a preventive effect in many types of cancers including BCa [152]. While a metaanalysis with data from 17 prospective study cohorts found no association between serum 25(OH)D<sub>3</sub> levels and BCa risk [293], a metaanalysis on BCa survival confirmed that serum 25(OH)D<sub>3</sub> concentrations have a strong dose-dependent inverse correlation with BCa mortality [294]. Prediagnostic 25(OH)D<sub>3</sub> levels modulated the expression of gene sets involved in proliferation, migration, and inflammation and were inversely associated with recurrence risk in patients with ER-positive tumors [295].

#### **4.2.7 Vitamin D-dependent gene expression in inflammatory and cancer-promoting pathways in the breast**

The COX-2/PGE<sub>2</sub> pathway is activated also in BCa, in both epithelial cells, lymphocytes, and tumor infiltrating macrophages [296]. PGE<sub>2</sub> induces aromatase expression, leading to higher estrogen (E<sub>2</sub>) levels in BCa cells, increasing proliferation, angiogenesis, and invasion [297]. Aromatase, an enzyme involved in estrogen synthesis, is overexpressed in BCa. In normal tissue, aromatase expression is driven by the glucocorticoid-stimulated promoter I.4 in the CYP19 gene, while in BCa, it is stimulated through the tumor-specific proximal promoter I.3 and II, in a cAMP-mediated way [298]. There is a strong correlation between aromatase activity and COX-2 expression in breast tumors as

PGE<sub>2</sub> is considered the most potent natural inducer of this promoter region. COX-2 inhibitors suppress also aromatase activity [299]. In BCa patients, COX-2 levels were higher than in normal breast tissue and were associated with low VDR expression and low serum 25(OH)D<sub>3</sub> levels [300]. In BCa cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> suppressed COX-2 and stimulated 15-PGDH expression leading to lower PG levels [166,301,302]. Treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> and the COX-2 inhibitor celecoxib inhibited expression of COX-2 in a cooperative way [303]. 15-PGDH is considered a tumor suppressor also in BCa [304], similarly as in CAC.

1,25(OH)<sub>2</sub>D<sub>3</sub> influences the activity of aromatase in a tissue-dependent manner. In BCa cells, it inhibited aromatase activity and reduced estrogen synthesis. 1,25(OH)<sub>2</sub>D<sub>3</sub> injections in mice with MCF-7 xenografts confirmed these *in vitro* results, by inhibiting the expression of aromatase in the newly developed tumors and the surrounding mammary adipose tissue. However, in osteosarcoma cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> had the opposite effect, inducing aromatase expression. This tissue-specific regulation makes 1,25(OH)<sub>2</sub>D<sub>3</sub> a selective aromatase modulator that can inhibit breast carcinogenesis, while protecting bone mineralization [301].

High-dietary intake of vitamin D and injected 1,25(OH)<sub>2</sub>D<sub>3</sub> had comparable tumor-reducing effects (50%–60%) in mice carrying MCF-7 xenografts. Both methods of vitamin D supplementation inhibited expression of aromatase, ER $\alpha$ , and the COX-2 pathway genes and reduced the level of estrone and estradiol. Vitamin D and 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited proliferation by increasing p21 and IGFBP-3 gene expression and induced apoptosis of the BCa xenografts by upregulating the proapoptotic Bax and downregulating the antiapoptotic Bcl-2 [261].

*In vitro*, 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analog BXL0124 prevented growth of BCa stem cell subpopulations shown to be responsible for BCa initiation [305]. Overexpression of VDR in CD133<sup>+</sup> cancer stem cells and treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> sensitized these to tamoxifen and inhibited spheroid formation by inhibiting Wnt/ $\beta$ -catenin signaling [306]. BXL0124 prevented the invasive capacity of cancer cells by inhibiting the expression of the stem cell marker CD44 that further repressed STAT3 signaling, which is responsible for BCa progression [307]. In MCF-7 BCa cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment inhibited nuclear translocation of the p65 subunit of the NF- $\kappa$ B, inhibiting this proinflammatory pathway [308]. In MCF12F normal breast cells, 25(OH)D<sub>3</sub> protected against cellular stress, oxidative stress, hypoxia, and apoptosis [309]. In mammary glands resected from mice and treated with carcinogens to induce precancerous lesions, treatment with 25(OH)D<sub>3</sub> had a

chemopreventive effect [310]. In the ER-negative BCa cell line MDA-MB-453, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the Ras/MEK/ERK signaling pathway [311]. For additional information, see also Chapter 88.

#### 4.2.8 Effect of vitamin D in inflammation-associated pancreatic cancer

Pancreatic cancer is an aggressive disease with high recurrence and low survival rate; it is the fourth leading cause of cancer-related deaths. Pancreatitis, the chronic inflammation of the pancreas, is a main risk factor for pancreatic ductal adenocarcinoma (PDAC). Pancreatic tumors have inflammatory elements, and cancer-associated fibroblasts are infiltrating the tumor stroma. The pancreatic stellate cells (PSCs) are quiescent fibroblast-like cells that become activated due to injury or inflammation and acquire tumor supportive features [312]. Genomic investigations revealed 12 essential overlapping molecular signaling pathways altered in this type of cancer [313]. The key molecules (KRAS, MAPK, EGFR, STAT, CCK-BR, VEGF, PDGFR, TGF- $\beta$ , SMAD4, MMPs, COX-2) involved in pancreatic tumorigenesis affect tumor development and progression and invasion, inhibition of apoptosis, metastasis, and angiogenesis [314]. The Wnt/ $\beta$ -catenin pathway is required for initiation of pancreatic carcinogenesis [315]. The known pathways linking inflammation and cancer, NF- $\kappa$ B, COX-2, STAT3, and Wnt/ $\beta$ -catenin, are activated also in pancreatic tumors [316–318].

The different cell types of the pancreas express the vitamin D system at different levels. In pancreatic cancer, however, this system becomes altered. The VDR is highly expressed in the endocrine islets but is barely detectable in the exocrine pancreas both in patients with chronic pancreatitis and in patients with PDAC [319]. The VDR is expressed also in the stroma of pancreatic tumors. In chronic pancreatitis patients, the expression of the degrading enzyme CYP24A1 was highest in the islets, and in adenocarcinomas, the tumor cells expressed more CYP24A1 than any other cell type. During PDAC development, the islets lose CYP24A1, while the transformed ductal cells upregulate CYP24A1 expression, impairing the antiproliferative effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> in these cells [319,320]. Moreover, silencing CYP24A1 inhibited proliferation and invasion of PDAC cells without affecting their migrating ability [320]. In a Canadian population, SNPs in CYP24A1, CYP2R1, vitamin D-binding protein, retinoid X receptor alpha, and megalin were significantly associated with the risk of developing pancreatic cancer [321]. The pancreata of *Vdr*<sup>−/−</sup> mice have periacinar and periductal fibrosis, demonstrating the importance of an intact vitamin D system for normal pancreatic function [312].

The correlation between vitamin D and pancreatic cancer has been controversial, and its putative beneficial role was not confirmed by all studies [322–326]. A meta-analysis found that pancreatic cancer patients with high blood 25(OH)D<sub>3</sub> levels had significantly improved survival, but no association was seen between 25(OH)D<sub>3</sub> levels and cancer risk [327]. Ecological studies found an inverse relationship between UVB radiation and pancreatic cancer risk [328]. Inflammatory biomarkers were increased in PDAC patients with vitamin D deficiency, independent of disease stage [329]. A nested case–control study in a cohort of men from Finland observed an unexpected association between high pre-diagnostic serum 25(OH)D<sub>3</sub> levels and a threefold increased pancreatic cancer risk [330]. These results could not be entirely confirmed in a cohort of male and female subjects from the United States, where high concentrations of serum 25(OH)D<sub>3</sub> were not associated with a higher risk of pancreatic cancer. In the Prostate, Lung, Ovarian, and Colorectal Cancer Screening Trial cohort, increasing serum 25(OH)D<sub>3</sub> levels associated positively with the risk of pancreatic cancer, but only in men with low sun exposure, or men with blood collected during fall or winter [331].

Studies on dietary vitamin D intake and pancreatic cancer are also inconclusive. Supplementation with 600 IU/day vitamin D was associated with lower risk of pancreatic cancer in a prospective study [332]; on the other hand, a pooled analysis of 14 cohorts found no association between vitamin D intake and pancreatic cancer [333]. A phase II trial with seocalcitol (EB1089), a vitamin D analog that inhibited growth of pancreatic cancer xenografts in vivo, found no antitumorigenic effects in patients with inoperable pancreatic cancer [334]. However, these patients had very advanced disease, and the level of VDR in the tumor was unknown. In another phase II trial, high-dose (0.5 µg/kg) calcitriol in combination with docetaxel moderately increased the time-to-progression in patients with incurable pancreatic cancer [335].

In the Ela1-TAg transgenic mouse model of pancreatic acinar cell carcinoma, vitamin D supplementation had no detectable effect on tumor development [336]. In contrast, in mouse models of acute and chronic pancreatitis, therapy with calcipotriol (a potent and non-hypercalcemic vitamin D analog) reduced fibrosis and inflammation [312]. In an orthotopic allograft PDCA model using immune-competent hosts, calcipotriol administration improved the efficacy of gemcitabine treatment, reducing tumor volume, tumor growth, and prolonged survival, compared with the gemcitabine treatment alone [312].

In several pancreatic cancer cell lines, VDR was essential for the survival of rapidly dividing cells, and depletion of VDR increased the sensitivity of the cancer cells

to gemcitabine treatment, suggesting that inhibition of VDR might provide a new way to enhance the efficacy of genotoxic drugs [337].

#### **4.2.9 Vitamin D–dependent gene expression in inflammatory and cancer-promoting pathways in the pancreas**

Very often the cytokines secreted by the stroma and the activated pancreatic stellate cells play an essential role in promoting cancer initiation in the pancreas [338]. Liganded VDR can ameliorate this process, reducing markers of inflammation and fibrosis [312]. In activated PSCs isolated from mouse models of acute and chronic pancreatitis, calcipotriol inhibited expression of cancer-signature genes, such as inflammatory cytokines and growth factors, such as WNT2B, WNT2A, IL6, and CCL2, through a cross-talk with the TGF-β/sonic hedgehog pathway [312]. In a coculture model of the pancreatic epithelial cell line (MIAPaCa-2) with activated human cancer-associated PSCs, activation of the stromal, but not epithelial, VDR inhibited expression of genes involved in proliferation, survival, epithelial-to-mesenchymal transition, and chemoresistance. Additionally, calcipotriol inhibited phosphorylation of STAT3 [312].

In in vitro and in vivo studies, both in pancreatic tissue and in xenografts, different 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs inhibited proliferation, migration, and invasion, and induced apoptosis, mainly by inhibiting the Akt pathway [339]. 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analog 19-nor-1α,25-(OH)<sub>2</sub>D<sub>2</sub> (paricalcitol) induced cell cycle arrest in G0/G1 phase and consequently inhibited proliferation of pancreatic tumor cells by upregulating the expression of p21 and p27 [340,341].

In pancreatic cancer cells, Arensman et al. identified a bidirectional feedback loop between vitamin D and Wnt signaling [342]. Activation of the Wnt pathway induced VDR expression, leading to significantly higher VDR levels in pancreatic cancer cell lines with high autocrine Wnt activity, compared with cells with low Wnt activity. Calcipotriol inhibited Wnt/β-catenin activity in the cells with high Wnt activity, by reducing the protein level of the Wnt coreceptor LRP6 [342]. The mechanism involves the direct transcriptional upregulation of the low-density lipoprotein receptor adaptor protein 1 (LDLRAP1). LDLRAP1 is required for endocytosis of LDL receptors and probably facilitates the internalization of LRP6 and its degradation through endosomal trafficking to lysosomes [342].

As vitamin D affects both the tumor and the stroma, it could support coupling stromal reprogramming with tumor-directed cytotoxic and immunologic drugs in innovative PDCA therapies. For more information, see also [Chapter 92](#).



#### 4.2.10 Effect of vitamin D in inflammation-associated hepatocellular cancer

Chronic liver disease and cirrhosis are a major global health concern. Hepatocellular carcinoma (HCC) has a complex molecular signature with two major determinants. Its etiology can be associated either with cirrhosis due to hepatitis infection, alcohol abuse, mutagenic agents, or metabolic disruptions or with different mutations in tumor suppressor genes or oncogenes (e.g., *p53*, *MYC*, *TGF- $\beta$* , *PTEN*, *EGFR*) that alter key carcinogenic signaling pathways (*VEGF*, *PDGF*, *EGF*, *IGF*, *HGF/c-MET*, *MAPK*, *mTOR*, *Wnt/ $\beta$ -catenin*) [343–345].

Low serum 25(OH) $D_3$  levels inversely correlated with the degree of liver dysfunction [346]. Vitamin D deficiency was linked also to inflammatory and metabolic liver diseases, including infection with hepatitis B and C virus (HBV/HCV) [347]. Severe vitamin D deficiency is common in patients with chronic hepatitis C (CHC). Higher 25(OH) $D_3$  levels were associated with less inflammation and less liver fibrosis in CHC patients [348]. Some of the effects of vitamin D are mediated by microRNAs that have proven to be VDR targets, such as miR-27, miR-125, and miR-155 [349,350]. In vitro 1,25(OH) $_2D_3$  treatment upregulated miR-375 and inhibited genes involved in EMT, such as YAP-1 and c-MYC [351]. In an Egyptian hepatitis C virus (HCV)–infected patient group, low vitamin D levels, serum IL13, and miR-135a correlated with unresponsiveness to antiviral treatment and high HCC risk [352].

Fibrosis, a progressive deposition of extracellular matrix components, is a typical signature of hepatic cirrhosis and involves activation of hepatic stellate cells (HSCs). VDR is expressed in HSCs, where it regulates hepatic fibrogenesis by preventing activation of profibrotic genes [353–355]. Activated HSCs provide a strong tumor-promoting microenvironment in hepatic cancer development. Similar to its effects in PSC, the vitamin D analog calcipotriol induces accumulation of lipid droplets in HSC, a sign of quiescence of stellate cells [356]. Paricalcitol, an 1,25(OH) $_2D_3$  analog, reduced inflammatory liver damage and ameliorated fibrosis in vivo [357]. In HSCs, the signaling adapter and autophagy substrate p62 is critical for the proper activity of VDR, namely for its heterodimerization with RXR. In hyperactivated HSCs, 1,25(OH) $_2D_3$  was unable to prevent fibrosis, probably due to the very low levels of p62, leading to reduced binding of liganded VDR to its target genes [356].

The high expression of CYP2R1 in liver cells from patients with chronic hepatitis C was associated with a high expression of VDR in infiltrated inflammatory cells and low portal inflammation, suggesting that vitamin D may control the inflammatory response in patients with this disease [358]. A SNP involved in the development of

many endocrine autoimmune diseases, the CYP27B1-1260 promoter polymorphism (rs10877012), was associated with sustained virological response rates in Caucasian patients with HCV genotypes 1, 2, and 3, receiving interferon- $\alpha$ -based treatment. Genotype AA of rs10877012 correlated with higher serum 1,25(OH) $_2D_3$  levels, compared with the AC or CC genotypes. Genotype CC was more common in the patients with HCV genotype 1 than in the healthy participants [359]. Further polymorphisms in CYP27B1 and CYP24A1 were associated with higher risk to develop nonalcoholic fatty liver disease (NAFLD) in China [360]. Indeed, NAFLD has been associated with vitamin D deficiency. Vitamin D status influences the severity of NAFLD by regulating not only the intestinal innate immunity but also the composition of the intestinal microbiota [361]. In an animal model of nonalcoholic steatohepatitis (NASH), an advanced form of NAFLD, vitamin D deficiency exacerbated the severity of the disease [362]. In patients with NASH, VDR levels were negatively associated with the activity score of NAFLD, supporting the involvement of VDR in the regulation of the inflammatory processes that develop during this disease [358].

The molecular actions of 1,25(OH) $_2D_3$  in hepatocellular carcinoma are less well understood [344]. 1,25(OH) $_2D_3$  treatment of HCC cell lines inhibited cell growth and induced expression of the cell cycle inhibitor p21. In parallel, 1,25(OH) $_2D_3$  decreased the expression of histone deacetylase 2, an enzyme usually overexpressed in HCC tumors, leading to inhibition of transcription of p21 [345]. In rats, high-dietary vitamin D intake reduced the level of the inflammatory markers IL1 $\beta$  and TNF $\alpha$  [363]. In a liver fibrosis model, calcipotriol reduced fibrosis and inhibited activation of HSCs by affecting the NF- $\kappa$ B pathway [354]. In a mouse model of HCC, 1,25(OH) $_2D_3$  treatment reduced tumorigenesis by inhibiting inflammatory cytokine secretion [364], while in the Smad3-deficient mice, it modulated signaling through TLR7 and the Wnt pathway [365]. A vitamin D $_2$  analog, doxercalciferol, given together with carnosic acid, increased HCC cell death through autophagy and apoptosis [366].

## 5. Conclusions

Vitamin D deficiency is prevalent, and the vitamin D system is compromised in many inflammatory conditions and cancers. It is not yet completely and convincingly demonstrated whether inadequate vitamin D level is a risk factor or a consequence of these diseases. As vitamin D deficiency should be avoided in any event, we need consensus what the adequate serum vitamin D levels should be and how to reach these levels, keeping



the potential effects on cancer risk and inflammation in mind.

Regardless of its extrinsic or intrinsic origin, cancer-related inflammation is a crucial process in tumorigenesis. It supports proliferation and survival of tumor cells, angiogenesis, migration, invasion, and metastasis; suppresses the adaptive immunity; and inhibits the response to treatment. Vitamin D is able to counteract most of these carcinogenic processes by complex, cell- and tissue-dependent molecular mechanisms, where  $1,25(\text{OH})_2\text{D}_3$  or synthetic vitamin D analogs activate gene expression and different signaling pathways in both VDR-dependent and independent manner. Although the preclinical studies support the antiinflammatory and antitumorigenic effects of vitamin D and of the vitamin D system, clinical trials have yielded controversial results on the influence of vitamin D and of its analogs on the prevalence and survival rate in patients with different types of (inflammation-associated) cancers. Whether this could be attributed, at least in part, to the tolerogenic effects of vitamin D, should be examined in more detail.

The antineoplastic action of vitamin D and its natural or synthetic analogs relies on induction of differentiation and apoptosis, inhibition of proliferation, invasiveness, and angiogenesis. These antitumorigenic effects might be weakened by the immunotolerogenic effects of  $1,25(\text{OH})_2\text{D}_3$  such as inhibition of effector T cells and upregulation of regulatory T cells [367]. Cancer immunotherapy needs a functional immune system, e.g., enhanced T cell immunity at the tumor site, to be effective. Therefore, the immunomodulatory properties of vitamin D and its analogs should be considered when designing clinical trials using these agents in treatment of cancer patients. Thus, there is vital need for new, more effective and selective  $1,25(\text{OH})_2\text{D}_3$  analogs that should be tested in faultlessly designed clinical trials.

## 6. Summary points

- Both the innate and adaptive immune system express VDR and the vitamin D–metabolizing enzymes almost ubiquitously.
- Inflammation affects  $1,25(\text{OH})_2\text{D}_3$  levels by regulating the expression level of the metabolizing enzymes CYP27B1 and CYP24A1, and of the VDR.
- Vitamin D ameliorates severity of chronic inflammations by regulating the innate and adaptive immune system.
- Vitamin D affects most hallmarks of cancer and prevents inflammation-associated cancers by regulating events downstream both the extrinsic and intrinsic inflammatory pathways.

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# Vitamin D actions in mammary gland and breast cancer

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## OBJECTIVES

- Present an overview of breast cancer biology as a framework for evaluating the role of the vitamin D pathway in the prevention and treatment.
- Review observational and interventional studies designed to address the impact of vitamin D on human breast cancer.
- Discuss the expression and function of the vitamin D pathway in breast cancer model systems.
- Highlight mechanisms by which vitamin D and its nuclear receptor modulate breast cancer.

## 1. Introduction

### 1.1 Overview of breast cancer

According to the Center for Disease Control's 2019 statistics [1], breast cancer is the most commonly diagnosed malignancy in US women with over 264,000 cases annually. The majority of women diagnosed already harbor invasive cancers with a high risk for the development of deadly metastases. Like many of the solid tumors common in populations living in industrialized countries, the incidence and mortality of breast cancer increase with age. In fact, 80% of diagnoses and almost 90% of deaths from breast cancer are in women over the age of 50. Between 1999 and 2019, the age-adjusted breast cancer mortality rate per 100,000 women has

declined from 26.6 to 19.4, an encouraging trend which has been attributed to enhanced screening, new treatment approaches, and prevention strategies. Despite these improvements, breast cancer kills over 42,000 women yearly in the US. Breast cancer is increasingly recognized as a global health problem, as data from the World Health Organization's Global Cancer Observatory indicate that breast cancer is the most prevalent cancer worldwide (with over 2.2 million cases diagnosed and over 680,000 deaths in 2020).

Risk factors and etiologic agents associated with breast cancer include those related to reproductive history (age at menarche, age at menopause, nulliparity, parity after age 30, hormone replacement therapy), genetics, radiation exposure, obesity, and diet. Vitamin D is one dietary factor that has been mechanistically linked to breast cancer etiology and is the focus of this chapter. Established tumor suppressor genes associated with familial breast cancer include the breast cancer susceptibility genes *BRCA1* and *BRCA2*, *p53*, and *ATM*; however, it is estimated that less than 10% of breast cancers can be attributed to inherited loss of function mutations in these genes. The vast majority of breast cancer cases are sporadic in nature although, as described below, several characteristic molecular changes have been linked with disease pathology and prognosis.

### 1.2 Subtypes of breast cancer and prognosis

Breast cancer collectively refers to several neoplastic diseases that arise in the mammary gland. Of the multiple cell types in the gland (epithelial cells, stromal fibroblasts, adipocytes, immune cells), it is the epithelial cells



that give rise to the common clinically relevant breast cancers. Within the mammary epithelium, carcinomas can originate from either the luminal cells or the basal cells of the ducts and lobules [2]. Ductal carcinoma is the most commonly diagnosed type, accounting for approximately 85% of all breast cancers. The development of invasive cancer from precursor lesions such as ductal carcinoma in situ (DCIS) is driven by genetic and epi-genetic changes within the epithelial cells and altered signaling between the epithelial cells and their microenvironment. The resulting carcinomas are highly heterogeneous and distinct subtypes have been identified based on histopathology and genomic profiling. Tumors that express the estrogen receptor alpha (ER, encoded by the *ESR* gene) and the progesterone receptor (PR, encoded by the *PGR* gene) account for 60%–70% of the breast cancer cases diagnosed in humans [3]. While less common, tumors lacking ER or PR and those with high expression of the growth factor receptor, HER2 (encoded by the *ERBB2* gene), are generally more aggressive with reduced survival rates.

Despite continued research, breast cancer is associated with a 20% mortality rate within the first 5 years after diagnosis. The severity of the disease reflects many factors including metastatic potential, hormone independence, drug resistance, and histological heterogeneity. Sites of breast cancer metastasis include regional lymph nodes, lung, liver, brain, and bone. The median survival of patients with distant metastases is 2–3 years. Development of bone metastases is particularly common with approximately 80% of patients with metastatic disease exhibiting skeletal involvement. Mechanisms underlying the establishment and progression of breast tumor growth in bone are poorly understood, although high rates of bone turnover appear to favor skeletal metastases.

Treatment of breast cancer is based on histopathological features such as grade, stage, and hormone/growth factor receptor status as evaluated at diagnosis. Lesions confined to the duct which are termed DCIS are routinely treated by surgical removal (lumpectomy or mastectomy) followed by radiation therapy and in some cases antiestrogen therapies. For invasive breast cancers, surgery is followed by systemic therapy which will vary based on tumor stage and molecular characteristics. Patients with ER+ and/or PR+ tumors typically receive hormone ablation therapies such as the antiestrogen tamoxifen or inhibitors of CYP19, the aromatase enzyme that drives the local synthesis of estrogen in the breast. For those women whose tumors overexpress the growth factor receptor HER2, targeted therapy with antibodies such as Herceptin (Trastuzumab) is standard, usually in combination with

chemotherapy. Patients whose tumors lack hormone and growth factor receptors, which are called triple-negative breast cancers (TNBC) are candidates for systemic chemotherapeutic regimens. Additional treatments that target cell cycle, DNA repair, and immune checkpoints may be employed for those with advanced disease or drug resistance. Newly identified molecular features and the presence of distinct oncogenic pathways are continuously emerging as significant therapeutic targets for all subtypes of breast cancer. As discussed in detail below, the presence of the VDR in the majority of breast tumors suggests it may represent a molecular target for therapy.

### 1.3 Molecular heterogeneity of human breast cancer

Within the last decade, intensive genomic profiling of human tumors has enabled detailed molecular characterization of breast cancers, leading to the identification of five major subtypes of invasive ductal carcinoma [4–8]. These five subtypes, which represent about 80% of all breast cancers, include Luminal A, Luminal B, Basal, HER2 positive, and Normal Breast-like [4]. Each subtype exhibits distinct profiles of hormone and growth factor receptors (ER, PR, HER2), cytokeratins, and proliferation markers. Most importantly, each tumor subtype has a characteristic prognostic outcome, with Luminal A tumors (which are ER+, PR+, and HER2– and usually express functional p53 protein) typically having a much better prognosis than the other types. Luminal B tumors tend to be ER+, PR+, and HER2+ and are usually of higher grade with more frequent *TP53* mutations than Luminal A tumors. The HER2 subtype, with a characteristic elevation of HER2 growth factor receptor signaling (usually due to genomic amplification), is negative for ER and PR and usually of high tumor grade. Basal tumors are predominantly ER–, PR–, and HER2– (and therefore usually classified as TNBC) and often express HER1 and/or cytokeratin 5/6 proteins and *TP53* mutations. Most breast cancers that develop in women with loss of function mutations in BRCA1 cluster with the basal molecular subtype whereas BRCA2 mutated tumors are more likely to be ER+ [9]. Although many trials have assessed the impact of nutrients, including vitamin D, on breast cancer risk and progression, few have been designed to stratify results by molecular subtype. This review will highlight the cumulative data on vitamin D actions in breast cancer while emphasizing the gaps in knowledge regarding its effects on specific molecular subtypes.

### 1.4 VDR, mammary cell lineages, and breast cancer cells of origin

The mammary gland contains multiple stem, progenitor, and differentiated cell types that maintain tissue homeostasis and function throughout the dynamic changes associated with puberty, pregnancy, lactation, and involution [10]. It has been suggested that the molecular heterogeneity of breast tumors might reflect distinct cells of origin [11]. That is, the initial target for transformation could be a mammary stem cell, a committed progenitor cell, or a differentiated luminal or basal epithelial cell, all of which would give rise to tumors with distinct features. Santagata et al. [12] mapped nuclear receptor expression during mammary gland lineage determination and reported that VDR was not expressed in early progenitor cells or differentiated basal cells, but was acquired in a subset of mature luminal cell types. Thus, the expression and function of the vitamin D pathway in a particular tumor will likely depend on its cell of origin [13].

A related issue is a recognition that human breast cancers contain “cancer stem cells” or “tumor initiating cells” that drive tumor initiation and/or progression. Advances in techniques to identify and isolate mammary stem cells support the concept that stem cell populations exist in human breast tumors and that analogous populations can be identified in certain murine tumors and human xenografts. Because stem cells theoretically give rise to the entire heterogeneous tumor cell population, therapies designed to target stem cells in tumors are predicted to effect more long-standing

remissions or cures. Recent data indicating that vitamin D signaling reduces the viability of breast cancer stem cells has generated considerable interest as discussed below.

## 2. Observational and interventional studies on vitamin D and breast cancer

### 2.1 Overview of population studies

Assessment of vitamin D status in relation to breast cancer or other age-related chronic diseases is complicated by difficulties in accurately assessing intake from food and supplements and in estimating the amount of vitamin D generated through sunlight exposure. Known confounders include lifestyle, latitude, pollution, sunscreen, skin pigmentation, genetics, body mass index (BMI), and age. For breast cancer, the influence of reproductive history, menopausal status, and the use of hormone replacement therapies are also critical modifiers of risk. Supplementation studies to assess cancer prevention require large numbers of participants and a long duration of follow-up to ensure sufficient cancer cases for stratification by cancer subtype. The ideal protocol would include an assessment of serum 25(OH)D at baseline and in response to supplementation. Despite these caveats, observational studies, meta-analyses, and supplementation studies have provided support for the beneficial effects of vitamin D in reducing cancer risk as discussed below and summarized in Table 88.1.

**TABLE 88.1** Vitamin D and breast cancer: observational data and intervention trials.

References	Study design	Outcomes
<i>UVR and breast cancer incidence</i>		
Hiller et al. 2020 [14]	Meta-analysis of 13 studies that evaluated self-reported solar UVR exposure in relation to breast cancer risk.	Breast cancer risk decreased for women spending $\geq 1$ h/d in the sun versus $< 1$ h/d. Evident for lifetime or usual adult exposure as well as exposure during adolescence.
Gregoire et al. 2022 [15] Sister study	Prospective population study of 48,450 US women; UVR exposure estimated with spatiotemporal kriging models based on enrollment address.	UVR exposure inversely associated with risk of ER– (but not ER+) breast cancer. Effect limited to non-hispanic white women not taking vitamin D supplements.
<i>25(OH)D and breast cancer incidence</i>		
Estebanez et al. 2018 [16]	Meta-analysis. 29 case–control studies; four cohort studies.	Inverse relationship between 25(OH)D and breast cancer incidence, especially in premenopausal women. Breast cancer incidence is not associated with dietary vitamin D, use of vitamin D supplements or 1,25(OH) <sub>2</sub> D.

*Continued*

**TABLE 88.1** Vitamin D and breast cancer: observational data and intervention trials.—cont'd

References	Study design	Outcomes
Song et al. 2019 [17]	Meta-analysis. 44 case–control studies; six cohort studies. Assessed dose–response relationship.	Breast cancer risk inversely related to serum 25(OH)D: 5 nmol/L increase of serum 25(OH)D was associated with 6% decrease in breast cancer risk.
<i>25(OH)D and breast cancer outcome</i>		
Vaughan-Shaw et al. 2017 [18]	Meta-analysis (all cancers) 38 studies for overall survival; 23 studies for disease progression.	For breast cancer, serum 25(OH)D level inversely related to overall survival (HR = 0.75) and disease progression (HR = 0.66). Limited evidence for genetic variability to affect survival.
<i>Vitamin D supplementation trials</i>		
Manson et al. 2019 [19] VITAL trial NCT01169259	Men (≥50 years), women (≥55 years). Vit D: 12,927; Placebo: 12,944. Oral vitamin D 2000 IU/d.	5 years vitamin D supplementation did not decrease incidence of new cancers. Not powered to stratify for specific cancer types.
Chandler et al. 2020 [20] VITAL trial NCT01169259	Men (≥50 years), women (≥55 years). Vit D: 3884; Placebo: 3959. Oral vitamin D 2000 IU/d.	Vitamin D supplementation decreased incidence of advanced and fatal cancers; limited to those with healthy BMI.
Peila et al. 2021 [21] WHI trial NCT 00000611	Postmenopausal women. Vit D: 16,753; Placebo: 16,601. Oral vitamin D (400 IU/d) with calcium (1000 mg). Intervention phase = 7.1 years; Postintervention phase = 13.8 years.	18.0% reduced risk of DCIS with vitamin D supplementation in the postintervention period (HR = 0.76).
Bischoff-Ferrari 2022 [22] DO HEALTH trial NCT01745263	Men & women ≥70 years. Oral vitamin D (2000 IU/d), omega-3 fatty acids (1 g/d), and/or exercise versus placebo 3 years follow-up.	Vitamin D alone reduced risk of total cancer (HR = 0.76). Cumulative cancer risk reduction with all 3 interventions (HR = 0.39).
Scragg et al. 2019 [23] VIDA trial ACTRN12611000402943	Men and women (50–84 years). VitD: 2513; Placebo: 2491. Oral vitamin D: Initial bolus dose of 200,000 IU, then 100,000 IU monthly versus placebo.	Monthly high-dose vitamin D supplementation did not decrease incidence of new in situ or invasive cancers over 3.3 years median follow-up.
Arnaout et al. 2019 [24] NCT01948128	Newly diagnosed breast cancer patients. VitD: 43; Placebo: 37. 40,000 IU/day 2–6 weeks prior to surgery.	High-dose vitamin D supplementation did not alter biomarkers of proliferation or apoptosis in primary tumor tissue.

## 2.2 Ultraviolet radiation (UVR) exposure and breast cancer risk

Observational studies have suggested that the incidences of several types of cancer are correlated with latitude and elevation, leading to the hypothesis that populations near the equator attain higher vitamin D status which delays or suppresses tumor development. As will be discussed in more detail in later sections, this hypothesis is supported by cellular and animal studies which have consistently demonstrated anti-tumor effects of 1,25dihydroxyvitamin D (1,25(OH)<sub>2</sub>D). Early large international studies demonstrated inverse correlations between incident solar radiation and cancer

rates as discussed in recent reviews [25,26]. In studies that have specifically assessed indices of cutaneous vitamin D synthesis (such as time spent outdoors, ambient/residential UVR, and/or skin pigmentation), about half have reported significant reductions in breast cancer incidence as a function of sunlight exposure, consistent with risk reduction in those with high vitamin D production. However, in some studies, these associations were limited to certain population subgroups and disease subtypes or dependent on the region of residence or skin type [27,28]. A large (>35,000 women) study which comprehensively analyzed sun sensitivity, time spent outdoors, and ambient UVR during five life

periods failed to detect a correlation between UVR and breast cancer incidence [29]. In contrast, a study of >38,000 Danish women indicated that after the age of 50 years, longer duration of UVR exposure ( $\geq 20$  years: OR = 0.83, 95% CI 0.75–0.92) and highest cumulative exposure (OR = 0.89, 95% CI 0.83–0.95) were inversely associated with risk [30]. A meta-analysis of 13 studies of self-reported measures of solar UVR exposure [14] reported a decreased risk of breast cancer for individuals spending  $\geq 1$  h per day in the sun during summer months over a lifetime versus those with lower exposure [pooled relative risk of 0.84]. Interestingly, exposure during adolescence (a critical developmental period for the breast) was more suggestive of a lower risk of breast cancer than exposure later in life. Data from the US Sister study (3500+ breast cancer diagnoses over 10+ years follow-up) which was not included in the meta-analysis demonstrated that residential UV exposure was inversely related to risk for ER– but not ER+ breast cancer [15]. The inconsistent results from various studies may be related to not only dose and duration of UVR exposure in relation to disease onset, but also genetics and physiological factors that modify an individual's ability to synthesize, store or utilize cutaneously-derived vitamin D. In addition to pigmentation, aging has long been known to reduce the epidermal synthesis of vitamin D [31] and mouse studies have recently demonstrated gender differences in UVB-induced vitamin D production related to androgen regulation of 7-dehydrocholesterol concentrations in the skin [32]. Therefore, there are likely multiple physiological and genetic factors that influence cutaneous vitamin D production. A better understanding of the regulation of vitamin D synthesis in the skin will be necessary to resolve the discrepancies in these population studies. In addition, when significant associations between UVR exposure and biological effects are observed, it remains important to distinguish the degree to which such associations result from vitamin D-dependent actions since UVR exerts vitamin D-independent effects as well.

### 2.3 Serum 25(OH)D and breast cancer risk

Observational studies designed to directly address the impact of vitamin D status from multiple sources, as measured by serum 25-hydroxyvitamin D (25(OH)D), on breast cancer incidence have also yielded mixed results. Several studies have supported the concept that high serum 25(OH)D is associated with decreased risk of breast cancer development [33–37], longer disease-free survival, or reduced mortality [38–40], while others have found significant correlations restricted to certain subgroups (i.e., postmenopausal women, younger subjects, ethnicity) and yet others

have demonstrated weak, nonsignificant inverse correlations or no associations [41–45]. A few of the most recent studies will be briefly reviewed here. Eliassen et al. [46] conducted a follow-up analysis of the Nurses Health Study that included >1500 breast cancer cases and controls, and while no association was found between 25(OH)D and breast cancer overall, there was a significant inverse correlation in women whose blood was sampled in summer. In a meta-analysis of 68 studies published between 1998 and 2018 [16], a protective effect of serum 25(OH)D (but not vitamin D intake or serum 1,25(OH)<sub>2</sub>D) on breast cancer risk was observed in both cohort studies (RR = 0.85, 95%CI:0.74–0.98) and case-control studies (OR = 0.65, 95%CI: 0.56–0.76). In a meta-analysis designed to evaluate the dose-response relationship between 25(OH)D and breast cancer risk, Song et al. calculated that an increase in serum 25(OH)D by 5 nmol/L was associated with a 6% decrease in breast cancer, with equal effects in pre- and postmenopausal women [17]. In contrast, some studies have found that the association between 25(OH)D and breast cancer risk was limited to pre-menopausal women [16]. Related to this observation, Cadeau et al. [47] conducted subgroup analysis of the French E3N cohort (>55,000 women and >2400 breast cancer cases) to evaluate potential interactions between vitamin D supplement use and hormone replacement therapy (HRT). While serum 25(OH)D was not measured, the use of vitamin D supplements was found to be protective against breast cancer in women who had taken HRT. Surprisingly, the opposite effect (increased risk) was observed with vitamin D supplementation in women who had never used HRT.

The relationship between vitamin D status and the risk of breast cancer in African Americans and other populations with a high frequency of vitamin D deficiency/insufficiency due to genetic or cultural factors has been understudied. In a multi-ethnic cohort, an inverse relationship between serum 25(OH)D and breast cancer was found in Caucasian women but not other racial groups including African-Americans [34]. In the Sister study, with a mean follow-up of 9.2 years, women with circulating 25(OH)D concentrations above the clinical cut point for deficiency (20.0 ng/mL) had lower breast cancer rates than women with concentrations  $\leq 20$  ng/, and the inverse association was strongest among Hispanic/Latina women, with a weaker association observed among Black/African American women [48]. However, the inverse association between the recent use of vitamin D supplements and breast cancer risk was similar in African American/Black and non-Hispanic White populations [49]. In a small case-control study of Iranian women, another ethnic population at high risk for vitamin D deficiency, subjects in the fourth quartile of serum 25(OH)D level



had threefold lower risk of developing breast cancer compared to those in the first quartile, but in this population, the relationship was observed in premenopausal but not postmenopausal women [50]. Prospective analysis of the Black Women's Health Study used a validated prediction model to assess cumulative serum 25(OH)D in relation to breast cancer. This data showed that 45% of participants were categorized as vitamin D deficient (<20 ng/mL) or insufficient (20–29 ng/mL) and that breast cancer risk significantly increased with decreasing quartile of predicted 25(OH)D<sub>3</sub> [51]. Further analysis of this population is of interest since it is not clear how well predictive models reflect actual serum 25(OH)D<sub>3</sub> levels [52]. Collectively, these selected studies highlight many of the factors that modify the relationship between vitamin D status and breast cancer including seasonality, ethnicity/race, menopausal status, and HRT use. These and other factors such as study size and analytical methods likely account for the diverse conclusions in population studies.

## 2.4 Serum 25(OH)D and breast cancer aggressiveness/survival

Shirazi et al. [53] assessed the association of vitamin D with breast cancer aggressiveness through analysis of serum 25(OH)D from >750 incident breast cancer cases and matched control subjects in the Malmo Diet and Cancer Study. They found that women with prediagnostic serum levels of 25(OH)D between 77 and 97 nmolar had the lowest risk of developing aggressive tumors (i.e., ER and PR negative, high mitotic index). Of note, women with serum 25(OH)D<sub>3</sub> <76 or >98 nmolar were significantly more likely to develop tumors with unfavorable prognosis, suggestive of bi-phasic effects of vitamin D status on disease progression. Further analysis of this cohort after mean follow-up of 13.1 years was conducted to assess the impact of prediagnostic serum 25(OH)D and tumor VDR expression [54]. Only patients with invasive breast cancer without bilateral disease or distant metastases and who did not undergo treatment were included (691 controls and 497 breast cancer cases). Women with VDR-positive tumors exhibited better survival especially when prediagnostic serum 25(OH)D was in the lowest tertile. Consistent with these reports, a systematic review and meta-analysis of vitamin D status and cancer survival (which included breast cancer cases), reported longer progression-free survival and reduced risk of death in those with high compared to low 25(OH)D levels [18]. Within the last several years, the adverse impact of vitamin D deficiency on survival of breast cancer patients has been confirmed in five

additional studies using various approaches [9,55–58]. Perhaps more clinically relevant, a beneficial effect of vitamin D status on disease recurrence after anti-endocrine therapy for ER+ breast cancer was reported by Lim et al. [59]. A striking increase in the incidence of late recurrences (particularly in regional lymph nodes, bone, and visceral sites) was observed in patients categorized as vitamin D deficient (serum 25(OH)D < 20 ng/mL) at the end of the standard 5-year course of endocrine therapy. Overall, the consistency of data linking low serum 25(OH)D with disease aggressiveness and poor survival warrant intervention studies to determine whether increasing vitamin D status after diagnosis will slow disease progression. Given there are >4 million breast cancer survivors in the US alone, a beneficial effect of vitamin D in this population would have a substantial translational impact.

## 2.5 Vitamin D and breast cancer subtypes

As discussed above, genomic studies have demonstrated that breast cancers are heterogeneous and that different subtypes exhibit distinct patterns of disease progression. It is likely that VDR expression or function and thus sensitivity to changes in vitamin D status may be subtype-specific, yet this has not rigorously been examined. The limited epidemiologic data that has been stratified by subtype is mixed. Yao et al. [60] conducted case-control and case series analyses and found that the relationship between serum 25(OH)D and reduced risk of breast cancer was strongest for high-grade, ER-negative, and triple-negative cancers. This finding is supported by the Malmo study cited above [53] which found a higher incidence of ER and PR tumors in women with low serum 25(OH)D. In contrast, Kim et al. [61] reported that low serum 25(OH)D was associated with poor prognosis only in women with the luminal subtype of breast cancer.

Qin et al. [62] conducted a case-control study to assess the association between vitamin D and breast cancer subtypes among African-American/black women, who tend to develop more aggressive forms of the disease. Evaluation of diet, supplement use, and sunlight exposure in 1724 cases (1213 ER+, 511 ER–, and 335 TNBC) and 1233 controls suggested that a moderate intake of supplemental vitamin D (up to 800 IU/day, but not >800 IU/day) was associated with decreased risk of several breast cancer subtypes, with effects that appeared strongest for TNBC. More sunlight exposure was also associated with decreased risk of both the ER– and TNBC subtypes in this population. These associations were not altered by menopausal status or obesity.

## 2.6 Vitamin D supplementation trials for breast cancer prevention

The gold standard tests for the effectiveness of cancer preventive agents are the large randomized controlled trials (RCT) in which healthy subjects receive either the proposed chemoprevention regimen or a placebo. Although many RCTs of vitamin D supplementation have been conducted, most have been designed to assess end points other than cancer and thus have not been sufficiently powered nor of long enough duration for meaningful results with respect to the development of a low incidence, heterogeneous disease that develops slowly such as cancer. The first large RCT designed to assess the impact of vitamin D supplementation on cancer end points was the Women's Health Initiative (WHI). This study recruited >30,000 postmenopausal women to a supplement containing calcium (1000 mg) and vitamin D (400 IU) or a placebo for an average duration of 7–11 years. This trial was confounded by the low dose of vitamin D utilized, the coadministration of calcium supplements, poor compliance, extensive pre-trial supplement use in the study population, and the freedom for trial participants to take additional personal supplements of up to 1000 IU vitamin D per day. The initial WHI results indicated no significant effect of vitamin D plus calcium supplementation on breast cancer or other endpoints such as colon cancer and cardiovascular disease. Subgroup and follow-up analyses of trial participants have yielded mixed results. One follow-up analysis [63] after a mean of 10 years that also analyzed dietary vitamin D indicated that higher intake of vitamin D was associated with a lower risk of premenopausal breast cancer (hazard ratio in the highest quartile compared to the lowest was 0.65). An inverse association between vitamin D intake and the incidence of large or poorly differentiated breast tumors was also detected among premenopausal women. However, no associations were identified in postmenopausal women. In another analysis of 15,646 women in the WHI cohort who were not taking personal calcium or vitamin D supplements at randomization, calcium plus vitamin D supplementation significantly decreased the risk of total cancers, all breast cancers, and invasive breast cancers by 14%–20% [64]. Recent analysis of the WHI study participants after 20+ years follow-up revealed a significant reduction in the incidence of DCIS in the calcium and vitamin D cohort [21], with the biggest effect observed in participants who also took personal vitamin D supplements during the intervention period. This later finding is noteworthy for two reasons. First, it suggests that vitamin D status may affect the earliest stage of breast cancer as DCIS is considered a preneoplastic condition. Second, the additive impact of personal supplement use in the intervention cohort indicates that the

low study dose (400 IU/day) did not optimize serum 25(OH)D for the prevention of early-stage cancer. Consistent with these findings, O'Brien et al. [49] reported a strong inverse association between the recent use of vitamin D supplements and DCIS in the Sister study. Collectively, these findings have enormous translational potential as more than 50,000 women are diagnosed with DCIS annually in the US alone [65].

In 2019, results from the Harvard-based VITAL trial of 20,000 US men (>50 years) and women (>55 years) randomized to daily supplements of 2000 IU vitamin D or placebo for 5 years were reported. Initial analysis of the VITAL study showed no significant effect of vitamin D supplementation on total cancer development, but a possible reduction in fatal cancers [19]. In secondary analysis [20] a significant reduction in metastatic and fatal cancers was found for those randomized to vitamin D compared with placebo (Hazard Ratio = 0.83). When stratified by BMI, there was a significant reduction for the vitamin D arm in incident metastatic or fatal cancer among those with normal BMI but not among those categorized as overweight or obese. The effect of vitamin D supplementation on advanced cancers was not modified by baseline serum 25(OH)D level or self-identified race category. Due to the relatively short duration of this trial, the number of cases was inadequate to stratify by cancer type, although the biggest effect of vitamin D supplementation appeared to be for the reduction of advanced/fatal prostate cancer.

In a smaller trial on cancer incidence (DO-HEALTH), 2157 European men and women over 70 years of age were randomized to either vitamin D (2000 IU/day), omega-3 fatty acids, an exercise program, or all three interventions [22]. After 3 years of follow-up, vitamin D supplementation significantly reduced the incidence of all cancers (Hazard Ratio = 0.76) and breast cancer (Hazard Ratio = 0.83). Further risk reduction was observed when vitamin D was combined with omega-3 or exercise and the lowest risk was in those assigned to all three interventions (Hazard Ratio = 0.26) [22].

Two additional supplementation trials are worth mentioning. The New Zealand-based ViDA trial [23] randomized ~5000 men and women (50–84 years) to monthly high-dose vitamin D (initial bolus dose of 200,000 IU followed by 100,000 IU monthly) or placebo for 3.3 years. No beneficial effect of vitamin D supplementation on new in situ or invasive cancers was observed over the relatively short time frame. It should be noted that many factors modify the impact of bolus doses [66] and their use is controversial [67]. Another recent trial [24] addressed whether high-dose vitamin D supplementation (40,000 IU/d) for newly diagnosed patients during the short time period between diagnosis and surgery would alter cell turnover in the breast

tissue. No significant differences in tumor cell proliferation or apoptosis were detected in breast tissues removed at surgery, possibly due to the unusually high dose, small number of patients, heterogeneity of disease, and/or variable duration of supplementation. However, additional studies of this nature are warranted to determine if the time period between diagnosis and surgery is a window of opportunity for vitamin D supplementation with respect to disease prognosis.

## 2.7 Summary of observational and intervention studies

As summarized in Table 88.1, recent studies have provided considerable support that low vitamin D status is associated with increased risk for breast cancer, more aggressive disease and reduced survival. It should be noted that while vitamin D deficiency is common in all breast cancer patient populations, it is particularly prevalent in those with triple negative/basal-like tumors, the most aggressive form of the disease [68–70]. Although progress has been made in clarifying the impact of vitamin D supplementation on breast cancer, continued follow-up of the cohorts described above (especially the VITAL population) is clearly warranted. Even in the absence of rigorous “proof” of the beneficial effect of supplemental vitamin D on breast cancer, correction of vitamin D deficiency in women at risk for, or living with, breast cancer should be standard practice.

## 3. Expression and function of the vitamin D pathway in normal and neoplastic breast cells/tissues

### 3.1 VDR expression and function in normal mammary gland

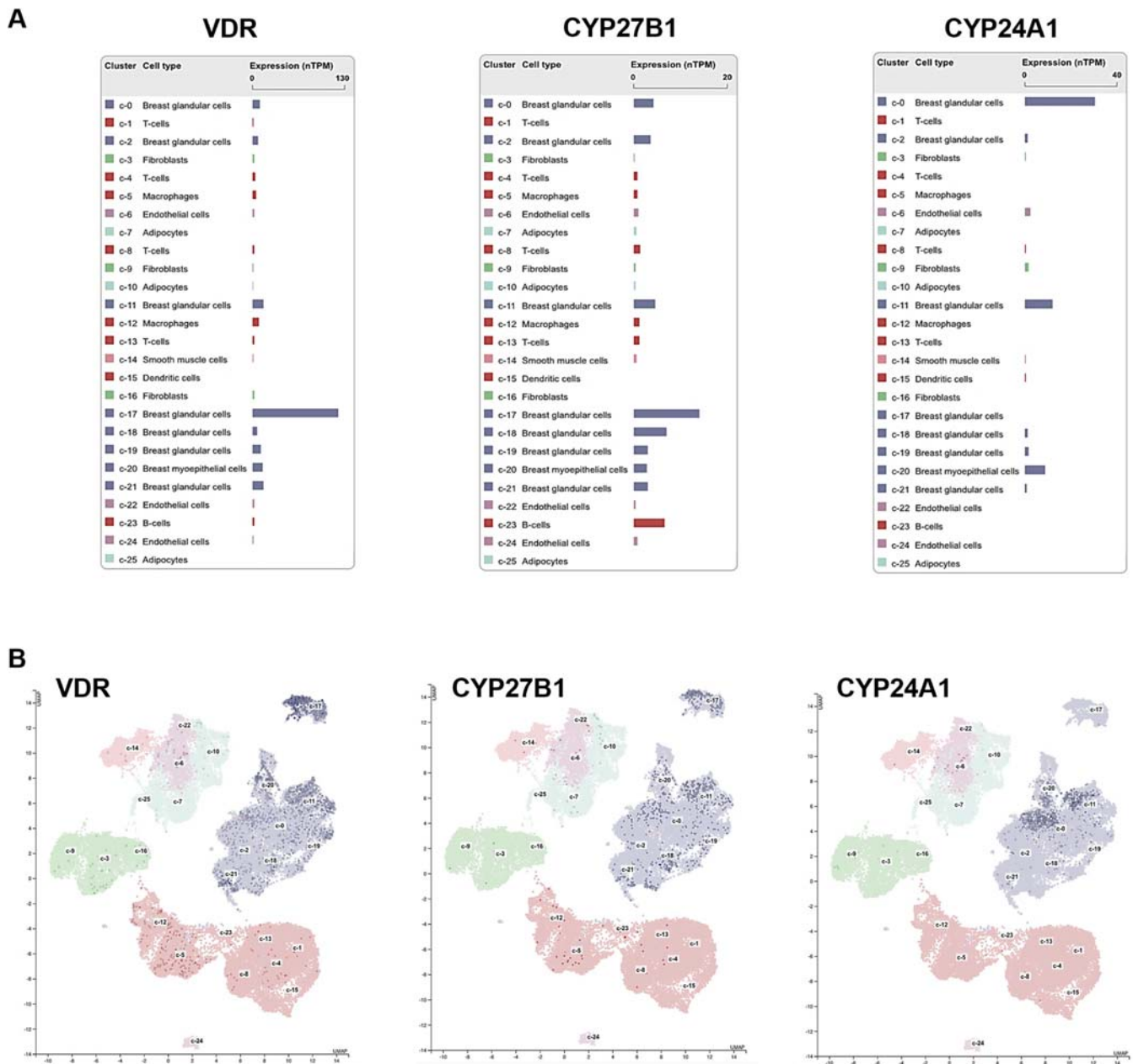
The VDR has been detected in rat, mouse, and human mammary glands and its expression is highest during puberty, pregnancy, and lactation [71,72]. High content immunohistochemistry of normal human breast epithelium demonstrated that VDR was expressed in nonproliferating, differentiated luminal cells and partially co-localized with ER [12]. We mapped VDR mRNA expression in various cell types of the normal adult human breast using a single-cell RNA-seq dataset [73]. As shown in Fig. 88.1A, VDR expression is highest in the eight cellular clusters representative of the epithelial compartment (highlighted in purple). VDR expression is particularly enriched in c-17, a cluster defined by expression of beta-casein (CSN2). As the major protein component of milk, beta-casein also binds and transports calcium into milk. Co-localization of VDR with CSN2 in secretory luminal epithelial cells is consistent

with the study of Santagata (12) cited above and suggests a role for VDR in lactation (further discussed below). VDR is detected at low levels in stromal and immune cell types (pink, red, and green clusters in Fig. 88.1A), consistent with published reports [74–76] and highlighting the potential complexity of 1,25(OH)<sub>2</sub>D signaling in the breast tissue environment.

Studies on the vitamin D pathway in the murine mammary gland have revealed an important role in the vitamin D pathway during development (Table 88.2). A distinct gradient of VDR protein expression was observed during the pubertal development of the mouse mammary gland, with weak VDR staining in proliferative populations and strong VDR staining in differentiated populations [72]. The role of the *Vdr* in mammary ductal morphogenesis was examined in *Vdr* knockout (VDRKO) mice fed high dietary calcium which normalizes fertility and neonatal growth [72]. During puberty, mammary glands from virgin VDRKO mice were heavier and exhibited accelerated growth, as evidenced by higher numbers of terminal end buds, greater ductal outgrowth, and enhanced secondary branching compared with glands from age- and weight-matched wild-type mice. In addition, glands from VDRKO mice exhibit enhanced growth in response to exogenous estrogen and progesterone, both in vivo and in organ culture, compared with glands from wild-type mice. 1,25(OH)<sub>2</sub>D inhibited growth and side branching of glands from wild-type mice but not VDRKO mice. More recent studies have implicated autophagy as a vitamin D-regulated pathway in the mammary gland [82]. Both dietary vitamin D and deletion of the VDR significantly modulated basal levels of autophagy in the murine mammary gland in the absence of hypercalcemia. These data provided convincing in vivo evidence that 1,25(OH)<sub>2</sub>D and the VDR impact ductal elongation, branching morphogenesis, and tissue homeostasis in the mammary gland.

Given the expression of VDR in multiple cell types in the gland, the effects of selective *Vdr* ablation in epithelial and adipose cells have been evaluated. Johnson et al. [79] reported that deletion of *Vdr* in either cellular compartment accelerated ductal morphogenesis in association with increased proliferation and decreased apoptosis within terminal end buds. VDR signaling in the mammary epithelium modulated estrogen- and progesterone-induced alveolar growth, as ablation of *Vdr* in this cell type resulted in precocious alveolar development. Ex vivo studies demonstrated that ligand-dependent VDR signaling in adipocytes inhibited mammary epithelial cell growth in part through 1,25(OH)<sub>2</sub>D-dependent production of the cytokine *IL-6* [79]. Subsequently, Matthews et al. [83] studied adult mice with adipose-specific *Vdr* deletion reared on high-fat diets and reported enhanced mammary





**FIGURE 88.1** Single cell analysis of VDR, CYP27B1, and CYP24A1 mRNA expression in human breast tissue.

epithelial density and branching relative to control animals. These data support the hypothesis that VDR in mature adipocytes alters the metabolic response to high-fat diets and exerts antiproliferative effects on the mammary epithelium. Collectively, these results suggest that vitamin D signaling contributes to growth inhibition of the mammary gland through independent actions in epithelial and stromal (adipose) compartments.

The role of VDR during lactation is poorly understood. Although *Vdr* remains highly expressed throughout pregnancy and lactation, neither glandular morphology nor milk calcium content was affected by

*Vdr* deletion in mice [77]. A caveat to these results is that VDRKO mice are typically reared on high-calcium diets which may facilitate calcium transport across mammary secretory cells in the absence of the receptor. A direct role for  $1,25(\text{OH})_2\text{D}$  in the regulation of calcium transport into milk was recently demonstrated in an in vitro model of secretory mammary epithelial cells [84].  $1,25(\text{OH})_2\text{D}$  increased the expression of genes encoding proteins involved in calcium and glucose transport (Calbindin D9K, PMCA1b, PMCA2b, GLUT1, and GLUT12) as well as the activities of several enzymes involved in these processes (hexokinase, CaMg



**TABLE 88.2** Animal studies on vitamin D pathway in mammary gland and breast cancer.

Model	Outcome
<b>Development, aging, spontaneous tumorigenesis</b> <b>Mice with targeted deletion of vitamin D pathway genes</b>	
Wild-type (WT) and <i>Vdr</i> knockout (VDRKO) mice maintained on rescue diet: mammary development and aging studies [72,77,78].	High VDR expression in differentiated epithelial cells of WT mice. Increased hormone-stimulated proliferation and branching in VDRKO glands in organ culture and in vivo compared to WT mice. Delayed glandular regression after lactation in VDRKO relative to WT mice.
Mammary epithelial- or adipocyte-specific <i>Vdr</i> KO: development study [79]	VDR in both adipose and epithelial cells functions to restrict pubertal glandular proliferation/development. Epithelial VDR (but not adipose VDR) functions to restrict alveologenesis during pregnancy. 1,25D induced secretion of IL-6 and leptin via adipose VDR ex vivo.
WT and <i>Cyp27b1</i> knockout (CYP27B1KO) mice: aging study [80].	Increased age-related spontaneous tumor burden in CYP27B1KO mice compared to WT mice. Prevented by either 1,25D or antioxidants, implying that lack of 1,25D enhanced oxidative stress and DNA damage. Mechanistic data implicated HGF and MET receptor in driving tumorigenesis.
Mammary epithelium specific deletion of <i>Cyp24a1</i> : Development study [81].	<i>Cyp24a1</i> deletion in mammary epithelium reduced proliferation and inhibited ductal budding, outgrowth, and branching (at puberty) and alveologenesis (in early pregnancy).
<b>Chemically induced mammary tumorigenesis</b> <b>Mice exposed to DMBA (dimethylbenzanthracene) develop mammary tumors that express ER and PR</b>	
WT and VDRKO mice fed high calcium rescue diet were evaluated for glandular morphology and mammary tumorigenesis after DMBA treatment [151].	Tumor incidence was near 100% in both WT and VDRKO mice, but VDRKO tumors were predominantly negative for ER and PR and exhibited trans-differentiation toward epidermis and hair. Glands from VDRKO mice showed impaired proliferative response to MPA stimulation compared to WT mice. Tumor histology in VDRKO mice was suggestive of <i>wnt</i> pathway activation.
Tumor incidence and burden were evaluated in mice fed AIN93G diets with standard or supplemental vitamin D prior to DMBA treatment [105].	Mice fed 25,000 IU/kg vitamin D had decreased tumor incidence and burden compared to those fed 1000 IU/kg. High dietary vitamin D inhibited pro-survival autophagy markers and increased the accumulation of p62. Tumor autophagy was reduced with vitamin D supplementation.
<b>Xenograft and allograft models</b> <b>Human or murine breast cancer cells injected into murine hosts at various sites to mimic primary tumor progression and/or metastatic colonization.</b>	
Mammary tumor cells isolated from DMBA-induced tumors generated in WT and VDRKO mice were grown as grafts in mice treated with EB1089 or exposed to UV radiation [133].	Chronic UV exposure increased serum 25(OH)D in tumor-bearing mice. EB1089 and UV exposure inhibited growth of xenografts containing <i>Vdr</i> <sup>+</sup> but not <i>Vdr</i> <sup>-</sup> tumor cells. Both treatments induced growth arrest and apoptosis in <i>Vdr</i> <sup>+</sup> but not <i>Vdr</i> <sup>-</sup> tumors.
MMTV-wnt1, 168-FARN, and 4T1 tumor grafts in immune competent Balb/c mice [135].	MMTV-wnt1 tumors grew faster in mice fed 25IU vitamin D/kg versus those fed 500 IU/kg. shRNA-mediated knockdown of <i>Vdr</i> in 168-FARN cells increased growth and metastasis while re-expression of <i>Vdr</i> reversed these effects. ID1 was identified as a VDR repressed gene in both vitamin D deficiency and <i>Vdr</i> knockdown studies.

**TABLE 88.2** Animal studies on vitamin D pathway in mammary gland and breast cancer.—cont'd

<b>Xenograft and allograft models</b>	
<b>Human or murine breast cancer cells injected into murine hosts at various sites to mimic primary tumor progression and/or metastatic colonization.</b>	
TM40D murine xenografts implanted into WT and CYP27B1KO mice [80].	Enhanced growth of xenografts in CYP27B1KO versus WT mice; with increased oxidative stress and DNA damage and tumor cellular senescence. Potential mediators HGF, c-MET, BMI1.
Orthotopic tumors derived from MDA-MB-231 TNBC cells expressing control or <i>CYP24A1</i> targeted shRNA [136].	Suppression of <i>CYP24A1</i> in MDA-MB-231 tumors reduced tumor weight, proliferation, and vascularity while enhancing apoptosis and necrosis. Gene expression profiling was conducted in MCF-7 and MDA-MB-231 cells upon <i>CYP24A1</i> silencing in vitro.
Comparison of skeletal metastases after intracardiac injection of MDA-MB-231 TNBC cells [181].	shRNA-mediated knockdown of <i>VDR</i> in injected tumor cells promoted epithelial-mesenchymal transition, cancer cell mobility (migration), and invasiveness, thereby facilitating skeletal colonization.
Growth and immune landscape in E0771 (murine luminal B-like) syngeneic xenografts in C57BL/6 mice [142].	Vitamin D supplementation (40 IU/mouse/day by gavage) slowed tumor growth, promoted CD8 <sup>+</sup> T cell infiltration, decreased systemic inflammatory cytokines, and reduced M1 macrophages in adipose depots. Obesity reversed the effects of vitamin D supplementation.
Growth of 4T1 (murine TNBC-like) xenografts in immune competent Balb/c mice [240].	Vitamin D supplementation (5 ug every other day) increased tumor burden which was abrogated by exercise training.
Growth and metastasis of 4T1 (murine TNBC-like) and E0771 (murine luminal B-like) xenografts in immune competent Balb/c and C57BL/6 mice [145,146].	Vitamin D supplementation tended to reduce growth of primary tumors composed of E0771 cells but not 4T1 cells. 1,25(OH) <sub>2</sub> D treatment or vitamin D supplementation increased lung metastasis of 4T1 but not E0771 xenografts. In contrast, vitamin D deficiency tended to increase metastasis of both E0771 and 4T1 xenografts. Refer to publication for comprehensive data on vitamin D metabolites in these models.
Combination of vitamin D analogs and an aromatase inhibitor [241].	PRI-2191 or PRI-2205 (noncalcemic vitamin D analogs) potentiated the antitumor effects of anastrozole in MCF-7 tumor-bearing mice. Combination treatment reduced aromatase gene expression and activity and down-regulated ER expression.
Combination of 1,25(OH) <sub>2</sub> D with tyrosine kinase inhibitors [214,242].	Additive or synergistic effects of 1,25(OH) <sub>2</sub> D with the kinase inhibitors dovitinib (cKIT, FGFR, VEGFR) and Ruxolitinib (JAK) in various breast cancer xenograft models:
Combination of 1,25(OH) <sub>2</sub> D with menadione (synthetic vitamin K) [243].	In murine transplantable triple negative breast tumor M-406, 1,25(OH) <sub>2</sub> D plus menadione reduced tumor growth rate and increased apoptosis more effectively than individual agents without weight loss.
Combination of 1,25(OH) <sub>2</sub> D with phytochemicals curcumin and resveratrol [237].	Antitumor effects of 1,25(OH) <sub>2</sub> D were enhanced in combination with curcumin or resveratrol in human MBCDF-T xenografts, primarily through inhibition of tumor angiogenesis.
<b>Transgenic models</b>	
<b>Engineered expression of hormones or oncogenes drive mammary tumorigenesis</b>	
<b>bLHβ-CTP mice:</b> Mammary hyperplasia and spontaneous tumors develop in response to chronic, systemic LH production [244].	LH-driven tumors had high <i>Vdr</i> expression. Tumor bearing mice were treated with VDR agonist EB1089. EB1089 inhibited tumor cell proliferation and reduced tumor burden in ~50% of treated mice.
<b>MMTV-NEU mice:</b> Mammary-epithelial expression of <i>Neu</i> oncogene induces stochastic tumors over prolonged time course [148].	Tumor bearing mice were treated with VDR agonist BXL0124. BXL0124 decreased tumor weight, incidence and multiplicity and inhibited ERBB2, ERK, and AKT signaling.

Continued

TABLE 88.2 Animal studies on vitamin D pathway in mammary gland and breast cancer.—cont’d

Transgenic models	
Engineered expression of hormones or oncogenes drive mammary tumorigenesis	
MMTV-NEU mice were treated with VDR agonist BXL0124 ± CDDO-Im (synthetic triterpenoid) either before or after tumor onset [147].	In prevention protocol, both BXL0124 and CDDO-Im delayed tumor development but the combination was most effective. In the therapeutic protocol, administration of the combination did not reduce tumor burden.
MMTV-PyMT mice: Mammary-epithelial expression of polyoma middle T antigen rapidly induces mammary tumors that metastasize to lung [149,245].	Low dietary vitamin D (25 IU/kg) accelerated tumorigenesis relative to standard diet (1000 IU/kg). Systemic perfusion with 25(OH)D or 1,25(OH) <sub>2</sub> D delayed tumorigenesis. Both 25(OH)D and 1,25(OH) <sub>2</sub> D accumulated in tumors. Vitamin D deficiency enhanced signaling through CXCL12/CXCR4 in lung metastatic niche and increased expression of pSTAT3 and ZEB1.
MMTV-PyMT mice were crossed with mice bearing mammary-specific deletion of <i>Cyp27b1</i> [150].	Targeted ablation of CYP27B1 in MMTV-PyMT mice accelerated mammary hyperplasia and tumorigenesis. NfκB and JAK-STAT signaling were increased in <i>Cyp27b1</i> ablated tumors. <i>Cyp27b1</i> ablation reduced tumor 1,25(OH) <sub>2</sub> D content.
MMTV-NEU mice: MMTV-NEU mice were crossed with VDRKO mice to generate WT, heterozygous (HET) and KO offspring bearing the <i>Neu</i> transgene [152].	Strong expression of VDR was detected in MMTV-NEU tumors and lung metastatic foci. Abnormal ductal morphology observed in VDRKO and VDR-HET mice. Poor long-term survival of VDRKO mice occurred due to weight loss and skin lesions. MMTV-Neu tumor incidence was increased in VDRHET versus WT mice.

ATPase, NaK ATPase). These data suggest the possibility that 1,25(OH)<sub>2</sub>D may enhance calcium transport into milk during lactation, but only when energy reserves are sufficient. Wagner’s group, who has pioneered studies on the effects of vitamin D supplementation during human pregnancy and lactation, has demonstrated correlations between maternal vitamin D status and milk composition, particularly with respect to cytokines and immune function [85,86].

A unique phenotype was observed in the mammary gland of aging VDRKO mice by Welsh et al. [78]. In contrast to the accelerated growth of the gland during puberty and pregnancy, atrophy of the mammary gland was observed in 12–16 month-old VDRKO mice. Relative to wild-type aged matched controls, VDRKO mice exhibited ductal ectasia of the primary mammary ducts, loss of secondary and tertiary branches, and atrophy of the adipose tissue due to activation of apoptosis. These morphological changes in the glands of aged VDRKO mice were associated with ovarian failure and reduced serum 17β-estradiol despite the maintenance of normocalcemia with the high calcium rescue diet. Subsequent

studies indicated that VDRKO mice exhibit a lean phenotype, resistance to obesity, and enhanced energy expenditure, suggesting that the mammary phenotype during aging may reflect long-standing systemic consequences of *Vdr* ablation [87]. It is unclear whether vitamin D status modulates the aging process in the human mammary gland.

### 3.2 Local metabolism of vitamin D in mammary gland

In addition to *Vdr*, the 25(OH)D metabolic enzyme *Cyp27b1* is detected in murine and human mammary tissue [77,88–90] suggesting that systemic 25(OH)D delivered to the mammary gland can be converted to the biologically active VDR ligand 1,25(OH)<sub>2</sub>D. In the single-cell analysis of a normal human breast, *CYP27B1* expression is enriched in c-17 (the epithelial cluster with the highest expression of VDR) and strongly co-localizes with VDR overall (Fig. 88.1B). Interestingly, *CYP24A1* expression (Fig. 88.1C) is not detected in c-17,

although its expression overlapped with *VDR* and *CYP27B1* in most other clusters. Of note, *CYP24A1* was one of only three genes expressed in luminal progenitor cells isolated from both human and mouse breast tissue, suggesting it may play a unique role in mammary cell lineage determination [91].

Developmentally, *Cyp27b1* expression was highest during mid-pregnancy in mice [77] suggesting that availability of 25(OH)D to the gland at this time may be especially critical. Lack of *Vdr* during pregnancy was associated with accelerated lobuloalveolar development, larger alveolar clusters, and accumulation of secretions in dilated lumens [77]. Although the impact of *Cyp27b1* ablation on glandular development during pregnancy has not been assessed, conditional deletion of *Cyp24a1* in mammary epithelial cells (which would be expected to enhance local 1,25(OH)<sub>2</sub>D accumulation) was associated with reductions in terminal end bud number, ductal outgrowth and branching during puberty as well as alveologenesis at early pregnancy [81]. Collectively, these murine studies support the concept that activation of VDR signaling by locally produced 1,25(OH)<sub>2</sub>D leads to physiologically relevant modulation of the mammary epithelium, particularly during periods of rapid development (puberty, pregnancy).

Consistent with data demonstrating expression of the vitamin D pathway in the mammary gland, *VDR* and *CYP27B1* were highly expressed in primary and immortalized cultures of nontumorigenic human mammary epithelial cells [89,90]. 1,25(OH)<sub>2</sub>D induced the known VDR target gene *CYP24A1* and exerted growth inhibitory effects indicating functional VDR in mammary epithelial cells. Dose-dependent growth inhibition was also observed in cells treated with 25(OH)D suggesting the existence of autocrine vitamin D signaling within the mammary epithelium. In vitro studies confirmed that incubation of mammary epithelial cells with physiological (nanomolar) concentrations of 25(OH)D led to temporal increases in 1,25(OH)<sub>2</sub>D detected in tissue culture media [89]. *CYP27B1* is also expressed in mammary adipocytes, which have been demonstrated to convert 25(OH)D to 1,25(OH)<sub>2</sub>D in organoid culture [76]. In response to 25(OH)D adipocytes secrete diffusible signals that inhibit morphogenesis of the adjacent ductal epithelium [76].

There is still uncertainty regarding how 25(OH)D, which circulates tightly bound to the vitamin D binding protein (DBP), is internalized by nonrenal cells. The presence of megalin and cubilin [92] in mammary epithelial cells indicates that these accessory proteins could mediate uptake of 25(OH)D-DBP complexes in the mammary gland as has been demonstrated for the kidney. Rowling et al. [92] demonstrated that normal breast epithelial cells and some breast cancer cells internalize DBP via megalin-mediated endocytosis. Given

the presence of *CYP27B1* in mammary adipocytes, it is worth noting that preadipocytes also express megalin and internalize 25(OH)D in culture [93]. However, the function of the megalin-mediated uptake pathway for the 25(OH)D-DBP complex in the intact mammary gland has yet to be confirmed.

### 3.3 Evidence for deregulation of the vitamin D pathway in breast cancer

Over 35 years ago, the recognition that VDR expression was detectable in breast cancers prompted extensive studies to determine whether targeting VDR in tumors would provide therapeutic benefit. VDR expression is retained in the majority of rodent breast tumors, human breast cancers, and established breast cancer cell lines [12,71,94,95]. However, some data suggest that receptor protein expression declines in highly aggressive tumors [94–96] and is deregulated by oncogenic transformation as described below [97–99]. It was therefore of interest to evaluate the frequency of genomic VDR changes in The Cancer Genome Atlas (TCGA) datasets of mutations, amplifications, deletions, and mRNA expression profiles in human breast cancers [100]. In this dataset of >450 cases, only 5% of invasive human breast tumors exhibited alterations in *VDR* sequence or expression [101]. However, when the *VDR* gene was altered, the most common change was a reduction in mRNA expression (deletions and mutations were not found). With respect to *VDR* expression in specific molecular subtypes, the highest frequency of alterations was in the Luminal B subtype (10.5% of tumors displayed reduced *VDR* mRNA expression) compared to 0%–3% for Luminal A, Basal, HER2, or Claudin-Low subtypes. The TCGA data showing unmutated *VDR* in the majority of human breast tumors are consistent with that of Santagata et al. [12] who used multiplex immunohistochemistry to map several nuclear receptors at the single cell level and confirmed that most human breast tumors express the VDR protein. In another survey of tumor VDR expression in >1000 patients, 42% of tumors had a negative or low expression, 32% had moderate expression and 25% had strong expression [102].

With respect to tumor VDR expression as a prognostic factor, an early study of 136 patients with primary breast cancer reported that women with VDR-negative tumors relapsed significantly earlier than women with VDR-positive tumors [103]. Consistent with this observation, Santagata et al. [12] reported that breast tumors with the highest expression of VDR, ER, and Androgen Receptor (AR) had the best prognosis [12]. In contrast, in the Al-Azhri study [102] there were no differences in overall survival, progression-free survival, or breast



cancer-specific survival by tumor VDR expression in the whole population or in various subgroup analyses. These discrepancies likely reflect the heterogeneity of VDR expression in breast cancer as well as the potential contribution of an individual's vitamin D status. With respect to the latter, the previously cited study by Huss et al. [54] found a significant association between tumor VDR expression and survival only for patients in the lowest tertile of serum 25(OH)D. As noted above, a subset of tumors express high levels of VDR, and in some datasets, high VDR expression correlates with poor, rather than better, survival [104]. Inherent differences in the populations studied, such as genetics, treatment history or vitamin D status may also contribute to these discrepancies. For example, using four publicly accessible genomic datasets comprising 581 cases, Li et al. [105] demonstrated that higher VDR expression in ER+ tumors predicted significantly longer recurrence-free survival, but only in patients treated with tamoxifen.

### 3.4 Mechanisms of vitamin D resistance in tumor cells

It should be noted that, even when tumors retain VDR expression, its antitumor functions are most likely attenuated. Mechanisms for ineffective VDR signaling include altered receptor function in the absence of mutation, reduced ligand availability, and/or mutation/deregulation of critical downstream VDR targets. Data on receptor expression derived from whole tumors may be somewhat misleading since cancer progression is driven by genetic instability and outgrowth of cells with advantageous mutations, such as the activation of oncogenes. In vitro studies have demonstrated that specific oncogenes can deregulate VDR expression. For example, a comparison of VDR expression in an isogenic mammary cell transformation model indicated that VDR expression and function were reduced by more than 70% in cells expressing SV40 and/or RAS compared to the parental cells [98]. Similar effects of SV40 and RAS on VDR activity have been demonstrated in other breast cancer model systems [97,106]. In addition, transcriptional repressors associated with cancer progressions such as SNAIL and SLUG have been shown to down-regulate VDR [99,107]. With respect to the deregulation of VDR function in gene expression, data from breast, bladder, and prostate cancer support the concept that cancer-associated alterations in transcriptional coregulators can also contribute to altered signaling by the 1,25(OH)<sub>2</sub>D-VDR complex [108,109]. Inhibition of VDR expression by miRNAs has also been demonstrated [110,111]. Overall, these data indicate

that abrogated expression and/or function of VDR may be common in certain subsets of cells within individual tumors that have sustained specific molecular genetic alterations. Clones that escape anticancer VDR signaling may eventually acquire sufficient additional properties leading to malignant progression.

In addition to genetic alterations and effects of oncogenes, VDR abundance in tumor cells may be affected by many physiological agents, including 1,25(OH)<sub>2</sub>D itself, estrogens, retinoids, and growth factors. Thus, cell sensitivity to 1,25(OH)<sub>2</sub>D may also reflect the activity of other hormone signaling pathways through their impact on VDR expression. In breast cancer, the regulation of VDR expression and activity by estrogens is likely to be clinically significant. Tumors that are positive for ER and PR express higher levels of VDR than ER-negative cells [102,112] and in vitro studies have demonstrated that estrogens, antiestrogens, and phytoestrogens regulate VDR in ER-positive breast cancer cells [113,114]. The degree to which estrogen status and commonly utilized synthetic or natural estrogens impact VDR expression in tumors is unclear and additional studies are necessary to assess the clinical relevance of these in vitro findings.

With respect to ligand availability, factors that enhance mammary cell catabolism of 1,25(OH)<sub>2</sub>D such as amplification of the *CYP24A1* gene [115] can also contribute to abrogation of VDR signaling. Analysis of TCGA datasets [101] confirms that a subset of human breast cancers (10%–13%) exhibit alterations in *CYP24A1*, with the most frequent changes being amplifications leading to upregulation at the mRNA level [104]. These data are consistent with the analysis of tumor samples which demonstrated amplification and/or higher protein expression of *CYP24A1* in breast tumors compared to adjacent normal tissue [90,94,115]. It is also worth noting that *CYP24A1* splice variants have been found in breast cancer cell lines [116], suggesting that distinct forms of the enzyme with altered properties may be expressed in tumors. *CYP24A1* alterations were found in all tumor subtypes, although amplifications were somewhat more frequent in Luminal B and HER2 tumors while increased *CYP24A1* mRNA was more common in Basal and Claudin-low tumors. Elevated *CYP24A1* expression correlated with reduced regression-free survival in the METABRIC dataset of >1900 breast cancers [104]. However, it is difficult to determine the extent to which increased *CYP24A1* expression contributes to disease progression since the amplified genomic region at 20q13 contains more than 300 distinct genes in addition to *CYP24A1* [104].

Given that normal mammary cells utilize 25(OH)D as substrate for local tissue generation of 1,25(OH)<sub>2</sub>D,

imbalanced expression of *CYP27B1* relative to *CYP24A1* could also theoretically contribute to the escape of tumor cells from anticancer VDR signaling. Oncogenic transformation of normal mammary epithelial cells was associated with reductions in *CYP27B1* expression and activity that were of sufficient magnitude to reduce cellular sensitivity to 25(OH)D approximately 100-fold [98]. However, clinical data on *CYP27B1* expression in breast cancer is inconsistent [90,94,117–119], and less than 2% of breast cancers annotated in TCGA datasets exhibit genomic alterations in *CYP27B1* [101]. In the METABRIC dataset cited above, *CYP27B1* alterations were almost exclusively up-regulations at the mRNA level [104]. Similar to *CYP24A1*, altered splice variants of *CYP27B1* have been detected in breast cancer cells [120,121] suggesting the possibility that forms of the enzyme with altered function could be expressed in breast tumors. Additional investigations are clearly needed to assess the clinical significance of *CYP24A1* and *CYP27B1* deregulation with respect to tumor progression.

## 4. Impact of vitamin D signaling in animal models of breast cancer

### 4.1 Overview of in vivo studies

Animal models of breast cancer have been extensively utilized to assess the effects of the vitamin D pathway on tumorigenesis. Early chemical carcinogenesis studies and trials with xenograft models formally established the efficacy of synthetic VDR agonists to prevent tumor development and reduce the growth of established tumors. In some cases, regression of existing tumors associated with apoptosis induction was reported. These data provided important proof of the principle that the antiproliferative and proapoptotic effects of 1,25(OH)<sub>2</sub>D that are consistently demonstrated in vitro could be achieved in vivo. Furthermore, synthetic analogs of 1,25(OH)<sub>2</sub>D were identified that elicited antitumor actions with minimal effects on calcemia (the most common and dangerous side effect of VDR-targeted therapies). A few animal studies also reported the efficacy of vitamin D analogs to reduce skeletal or other metastases and improve survival [122,123]. Please see Table 88.2 for a summary of newly published animal studies on the vitamin D pathway and breast cancer, some of which are highlighted below. Efforts have been focused on the use of knockout and transgenic models to assess mechanistic questions, the ability of dietary vitamin D to modulate tumor development and progression, and the promise of combination therapies.

### 4.2 Efficacy of vitamin D compounds to inhibit chemically induced carcinogenesis

In vivo, both high dietary vitamin D [105,124] and treatment with synthetic VDR agonists [105,125–129] inhibited the development of mammary tumors induced by chemical carcinogens such as MNU and DMBA. These tumors are typically estrogen-responsive and were shown to express VDR [130] suggesting the possibility that vitamin D compounds acted directly on tumor cells. The demonstration that VDR agonists including 1,25(OH)<sub>2</sub>D reduced the incidence of preneoplastic lesions induced by DMBA in mouse mammary gland organ cultures supports this suggestion [88,131]. Mechanistic studies in chemically-induced carcinogenesis models suggested that vitamin D exerted tumor-suppressive effects during the promotion and/or progression phases as opposed to the initiation phase [132].

### 4.3 Studies on VDR agonists, diet, and UVR in tumor graft models

Tumor grafts (both syngeneic and nonsyngeneic) have frequently been used to assess the in vivo effects of vitamin D signaling on breast tumorigenesis. Nonsyngeneic models include grafts of established cancer cells (usually human) into immune-deficient mice whereas syngeneic models involve grafting of murine cells into immune-competent host mice of identical genetic background. Numerous synthetic vitamin D analogs, including EB1089 and BXL0124, effectively inhibited the growth of ER+ (MCF-7) and ER– (MDA-231, MCF10DCIS.com, SUM159PT) tumors grafted in nude mice [123,128,133,134]. EB1089 also reduced the extent of skeletal metastases (total number of bone metastases, surface area of osteolytic lesions, and tumor burden within bone) and enhanced survival after intracardiac injection of MDA-MB-231 human breast cancer cells [122].

The absolute requirement for VDR in tumor responses to vitamin D-based therapies was first demonstrated in a study of EB1089-treated nude mice bearing xenografts of mammary tumor cells derived from wild-type or VDRKO mice [133]. Only tumors composed of VDR-positive cells from wild-type mice responded to EB1089 indicating that VDR in the mouse stroma or other tumor-infiltrating cells is not sufficient to mediate anticancer signaling in the context of a xenografted tumor. A novel aspect of this study was the demonstration that exposure of tumor bearing mice to UVR elevated serum 25D and reduced the growth rate of VDR-positive but not VDR-negative tumors [133]. Williams et al. [135] reported that shRNA-mediated

knockdown of *Vdr* in 168-FARN murine mammary tumor cells resulted in tumor grafts that grew faster and metastasized more readily than their *Vdr*-expressing counterparts. Importantly, these effects were reversed when *Vdr* was restored into *Vdr* knockdown cells. The effects of both VDR signaling and dietary vitamin D manipulation in this study were mechanistically linked to the regulation of the *Id1* oncogene. A suppressive role of VDR in metastatic spread was reported by Zhang et al. [96] who demonstrated that *Vdr* overexpression in 4T1 murine mammary tumor grafts suppressed lung colonization relative to that of control 4T1 grafts with low *Vdr* expression, and this effect was attributed to suppression of  $\beta$ -catenin by VDR. In MDA-MB-231 TNBC cells, VDR knockdown promoted epithelial-mesenchymal transition, migration, invasion, and skeletal colonization. Expression of vitamin D metabolic enzymes can also impact tumorigenesis. For example, allografts of TM40D mammary tumor cells grew more rapidly in *Cypb27b1* KO mice compared to wild-type mice, effects that were attributed to elevated oxidative stress, DNA damage, and senescence and were prevented by administration of 1,25(OH)<sub>2</sub>D but not 25(OH)D [80]. Consistent with these observations, shRNA-mediated inhibition of *CYP24A1* expression reduced the growth of TNBC xenografts through suppression of proliferation and angiogenesis in conjunction with activation of apoptosis and necrosis [136]. Collectively, this series of studies clearly demonstrated that VDR expression and availability of its ligand 1,25(OH)<sub>2</sub>D impact oncogenic signaling and tumor behavior in vivo.

Several studies have investigated whether dietary vitamin D manipulations alter the growth of breast xenografts. Swami et al. [137] demonstrated that increasing dietary vitamin D from 1000 IU/kg diet (rodent standard) to 5000 IU/kg diet significantly reduced the growth of established MCF-7 xenografts with a potency equivalent to that of treatment with 1,25(OH)<sub>2</sub>D [137]. This group also demonstrated that vitamin D signaling exerted inhibitory effects on aromatase gene expression and activity leading to reduction in estrogen-stimulated tumor growth. Supplemental dietary vitamin D also abrogated the tumor-promoting effects of obesity in a syngeneic graft model with Wnt1 cells [138]. Conversely, dietary vitamin D deficiency enhanced the growth of *Wnt1*-driven tumor grafts [135,139]. In a metastasis model in which either MCF-7 or MDA-MB-231-TxSA breast cancer cells were injected directly into the tibia of nude mice, vitamin D deficiency sufficient to elevate PTH and enhance bone turnover promoted the development of larger osteosclerotic lesions compared to those of vitamin D sufficient mice [140,141].

The possibility that vitamin D signaling affects anti-tumor immunity has been assessed in immune-competent models with murine allografts. Karkeni et al. [142] demonstrated that vitamin D supplementation promoted infiltration of CD8<sup>+</sup> T cells into E0771 allografts, decreased systemic inflammation, and reduced M1 macrophages in association with a reduction of tumor burden. Interestingly, the effects of vitamin D supplementation were abrogated by obesity. In a comprehensive series of studies that compared several allograft models, vitamin D supplementation had divergent effects on tumor growth, metastasis, blood flow, immune cell populations, systemic cytokines, and fibroblast phenotype [143–146]. These studies demonstrated that both tumor cell heterogeneity and host genetic background influenced the response of tumor-bearing mice to dietary vitamin D.

#### 4.4 Effect of dietary vitamin D and/or VDR agonists on transgenic models of breast cancer

The first demonstration that activation of vitamin D signaling could reduce tumorigenesis in a genetically engineered model of breast cancer was reported in 2005. This study utilized luteinizing hormone (LH) over-expressing mice, a model of chronic ovarian stimulation which leads to hormone-responsive mammary tumors with abundant VDR expression. Treatment with the low-calcemic vitamin D analog EB1089 decreased proliferation of mammary epithelial cells in preneoplastic glands and reduced the growth rate of a subset of tumors. Subsequent studies in the MMTV-NEU mouse model which mimics the HER2 subtype of human breast cancer demonstrated that the Gemini analog BXL0124 inhibited tumor growth and down-regulated oncogenic pathways driven by HER2 (such as ERK and AKT). Importantly, tumor growth inhibition was only observed when BXL0124 treatment was initiated prior to the development of palpable tumors suggesting that VDR signaling may become corrupted with tumor progression in this model [147,148].

Two recent studies evaluated the role of local generation of 1,25(OH)<sub>2</sub>D in transgenic mice that express the Polyoma Virus middle T antigen in the mammary gland (MMTV-PyMT mice). Tumors in this model are driven by the activation of numerous oncogenic pathways including MAPK, PI3K, and PLC- $\gamma$ . Tumor development was accelerated in MMTV-PyMT mice fed a low (25 IU/kg) vitamin D diet relative to those fed 1000 IU/kg vitamin D [149]. Conversely, systemic administration of 25(OH)D or 1,25(OH)<sub>2</sub>D delayed tumor appearance and decreased lung metastasis in this



model. Perfusion of mice with 25(OH)<sub>2</sub>D resulted in the accumulation of 1,25(OH)<sub>2</sub>D in tumors, supporting the idea that conversion of 25D to 1,25(OH)<sub>2</sub>D by tumor CYP27B1 occurred. In further support of this idea, the deletion of *Cyp27b1* in the mammary epithelium of MMTV-PyMT mice accelerated mammary tumorigenesis and up-regulated markers of cell proliferation, angiogenesis, cell cycle progression, and survival [150]. Oncogenic pathways including AKT, NF-κB, and STAT3 were increased in *Cyp27b1*-ablated tumors compared with nonablated controls.

#### 4.5 Effect of *Vdr* ablation on mammary carcinogenesis

In a follow-up to the rodent chemical carcinogenesis studies cited above, the effect of *Vdr* ablation on DMBA-induced mammary tumorigenesis was assessed. Zinser et al. [151] reported that VDRKO mice (maintained on the rescue diet to prevent hypocalcemia) exhibited an increased incidence of mammary gland hyperplasia and a higher percentage of hormone-independent tumors with squamous differentiation after treatment with DMBA than wild-type mice. In the transgenic MMTV-NEU mouse model of mammary cancer, mice heterozygous for *Vdr* exhibited shortened tumor latency and increased tumor incidence compared to wild-type mice [152]. Another study examined the effect of *Vdr* ablation in mice expressing the RON oncogene in the mammary epithelium (MMTV-RON mice), which develop mammary tumors associated with hyperactive β-catenin signaling. On the MMTV-RON background, mice homozygous or heterozygous for *Vdr* exhibited significantly longer times to palpable tumor formation compared to those with complete *Vdr* ablation [153]. Furthermore, *Vdr* deletion significantly increased the number of metastases in the lung and liver relative to that of mice homozygous or heterozygous for *Vdr*. The ability of VDR to suppress RON-mediated tumorigenesis was mechanistically linked to the induction of DKK-1 (a Wnt pathway inhibitor) and attenuation of β-catenin signaling by 1,25(OH)<sub>2</sub>D [153].

### 5. Genomic profiling of VDR agonists in breast cancer model systems

#### 5.1 Overview

Screening for molecular changes induced by 1,25(OH)<sub>2</sub>D or vitamin D analogs in various breast cancer model systems has identified a broad range of downstream VDR targets. Cell biology studies generally focus on a few targets to mechanistically assess their involvement in tumor responses to VDR agonists. In contrast,

genomic profiling typically identifies hundreds of vitamin D-regulated genes and allows for predictions of how these gene alterations may affect cellular pathways. While *CYP24A1* is commonly identified in microarray studies as the most highly up-regulated gene in 1,25(OH)<sub>2</sub>D-treated cells, other target genes vary greatly depending on the model system. Here we will focus on the few comprehensive genomic studies that have allowed for unbiased identification of vitamin D-responsive pathways in normal mammary cells and those derived from various types of breast cancer [74,154–159].

#### 5.2 1,25(OH)<sub>2</sub>D regulated genes and enriched pathways in nontumorigenic mammary epithelial cells

To identify potential mediators of vitamin D actions in cancer prevention, microarray profiling in nontransformed mammary epithelial cells (hTERT-HME1 cells) treated with 100 nM 1,25(OH)<sub>2</sub>D for 24 h was conducted [159]. It was previously shown that the biological responses of hTERT-HME1 cells to 1,25(OH)<sub>2</sub>D are highly similar to that of primary cultures of human breast epithelial cells indicating that the immortalization process per se does not affect VDR expression or function [89]. Treatment with 1,25(OH)<sub>2</sub>D significantly altered the expression of over 450 entities (65% up-regulated/35% down-regulated) in hTERT-HME1 cells. The top up-regulated genes included *CYP24A1*, *CD14*, *ITGB3*, *SLC1A1*, *DHRS9* and *BMP6*, while the top down-regulated genes included *KDR*, *BIRC3*, *RGS2*, and *GLUL*. These differentially expressed genes were associated with the enrichment of 42 biological pathways including senescence and autophagy, cell cycle checkpoints, and TGFβ signaling. Enrichment of these pathways is consistent with reports that longer-term incubation of hTERT-HME1 cells with 100 nM 1,25(OH)<sub>2</sub>D induces growth inhibition [89] and that TGFβ inhibits mammary epithelial cell growth [160]. Additional tumor suppressive pathways enriched in this gene set included those related to oxidative stress, Nrf2 signaling, cell adhesion, immune responses, and cellular metabolism as well as pathways related to distinct differentiated phenotypes (adipogenesis, angiogenesis, osteogenesis, and osteoclastogenesis). A subset of putative VDR target genes (*ITGB3*, *GLUL*, *KDR*, *BIRC3*, *SLC1A1*, *CD14*, *BMP6*, *IL1RL1*, *IL33*) was validated as 1,25(OH)<sub>2</sub>D-responsive in two independently derived telomerase immortalized HME cell lines [159,161] and the effects of 1,25(OH)<sub>2</sub>D were consistent with the initial array data. Thus these genes appear to be bonafide VDR targets in nontransformed mammary epithelial cells. It is worth noting that regulation of these



genes in response to  $1,25(\text{OH})_2\text{D}$  was highly discordant in nontumorigenic MCF10A cells, which are commonly used as a model of “normal” mammary epithelial cells and thus would be expected to respond comparably to the telomerase immortalized HME cells. Only two down-regulated genes (*KDR* and *BIRC3*) were similarly altered by  $1,25(\text{OH})_2\text{D}$  in hTERT-HME1 and MCF10A cells. Others that were down (*GLUL*) or up (*ITGB3*, *SLC1A1*) regulated by  $1,25(\text{OH})_2\text{D}$  in the immortalized HME cells were unaffected in MCF10A cells [159,161]. These data suggest that MCF10A cells, despite their nontumorigenic phenotype, may not be an appropriate model for the assessment of  $1,25(\text{OH})_2\text{D}$  actions in normal breast epithelial cells. Examination of the effects of  $1,25(\text{OH})_2\text{D}$  on several breast cancer cell lines also demonstrated considerable diversity in the regulation of these candidate genes. The discordance in gene regulation between nontumorigenic and cancerous cells was not due to reduced VDR sensitivity as  $1,25(\text{OH})_2\text{D}$  comparably induced *CYP24A1* (700–900-fold) in the various cell lines. In addition, the ability of  $1,25(\text{OH})_2\text{D}$  to alter expression did not obviously correlate with the basal expression of genes in these cell lines.

### 5.3 Genomic profiling in breast cancer cell lines

In the first genomic profiling of vitamin D actions in breast cancer cells, Feldman's group compared gene expression in ER+ MCF-7 cells and ER– MDA-MB-231 cells treated with 100 nM  $1,25(\text{OH})_2\text{D}$  for 24 h [162]. Due to the limited nature of these first-generation arrays, which were comprised of 2000 cancer-related genes, direct comparisons with newer datasets from whole genome profiling are not appropriate, but comparisons between the two cell lines is of interest. The study found differential expression of 62 genes in MCF-7 cells (47 up/15 down) as compared to 20 genes in MDA-MB-231 cells (10 up/10 down), with only seven genes commonly altered in both cell lines. The larger number of  $1,25(\text{OH})_2\text{D}$  regulated genes in MCF-7 cells was consistent with higher *CYP24A1* induction in these cells (52-fold) compared to MDA-MB-231 cells (5.5-fold). Significantly regulated genes in MCF-7 cells treated with  $1,25(\text{OH})_2\text{D}$  included *RBL2*, *CTNNA1*, *RAD23B*, *NCOA4*, *BMP5* and *IFNG* (up) and *CEACAM1*, *CDH6*, *IL13*, *IL1R2* and *ESR* (down). Highly modulated genes in MDA-MB-231 cells included *CASP4*, *NF1B*, *ITGAV*, *TXNRD1* and *TGFB2* (up) and *ANGPT1*, four *MMPs* (12, 10, 7, 1) and *PRKD1* (down). Although pathway analysis was not conducted, many of the  $1,25(\text{OH})_2\text{D}$  regulated genes in MCF-7 cells were linked to growth factor signaling, cell cycle, apoptosis, and immune responses, whereas genes related to disease progression (i.e., invasion and

angiogenesis) were more highly regulated in MDA-MB-231 cells.

Another array study compared the effects of 12 h treatment with 1 nM RO3582 (a Gemini  $1,25(\text{OH})_2\text{D}$  analog) in a model of premalignant (MCF10AT1) and malignant (MCF10CA1a) disease [156]. Consistent with the results in MCF-7 versus MDA-MB-231 cells [162], the less malignant MCF10AT1 cells displayed more significant changes in gene expression than the invasive MCF10CA1a cells (391 vs. 156, respectively). Despite the differences in sensitivity, about 55% of the genes altered in MCF10AT1 cells were similarly altered in the MCF10CA1a cells. The similarity in response is likely due to the common genetic background of these two cell lines, as both are derivatives of the MCF10A cell line discussed above.

Simmons et al. [159] reported whole genome profiling of MCF-7 cells (a model of luminal breast cancer) treated for 24 h with 100 nM  $1,25(\text{OH})_2\text{D}$ . In this analysis, 249 entities were significantly altered by  $1,25(\text{OH})_2\text{D}$  (153 up-regulated/96 down-regulated). The top up-regulated genes included *CYP24A1*, *KLK6*, *TRPV6*, *CP*, and *MERTK*, while the top down-regulated genes included *PMP22*, *CTGF*, *CLDN1*, and *ILIR1*. Pathway analysis of this MCF-7 dataset indicated enrichment of genes in 31 pathways, eight of which (senescence and autophagy, TGF $\beta$  signaling, endochondral ossification, adipogenesis, nuclear receptors in lipid metabolism/toxicity, oncostatin M signaling, integrated pancreatic cancer pathway, and integrin-mediated cell adhesion) were common to those enriched in the nontumorigenic hTERT-HME1 cell gene set discussed above. Perhaps more relevant for the disease process were the high-scoring cancer-relevant pathways altered by  $1,25(\text{OH})_2\text{D}$  in MCF-7 cells but not in hTERT-HME1 cells which included Phase I metabolism, apoptosis modulation and signaling, estrogen/tamoxifen metabolism, and ATM signaling.

Another publicly available dataset was reported by Goeman et al. [163] who utilized RNA-Seq to generate a list of differentially expressed genes in SKBR3 cells (a model of HER2+ breast cancer with mutant p53) treated for 6 h with 100 nM  $1,25(\text{OH})_2\text{D}$ . Simmons et al. [159] compared this dataset with their data from  $1,25(\text{OH})_2\text{D}$ -treated hTERT-HME1 and MCF-7 cells and identified 17 genes that were altered by  $1,25(\text{OH})_2\text{D}$  in all three breast-derived cell lines, however only 11 of these genes were regulated in the same direction (9 up, 2 down). Additional comparisons identified 26 genes coregulated by  $1,25(\text{OH})_2\text{D}$  in the two cancer cell lines (SKBR3 and MCF-7 cells) and 20 genes coregulated by  $1,25(\text{OH})_2\text{D}$  in SKBR3 and hTERT-HME1 cells. The full lists of gene changes are available in the publication by Simmons et al. [159].

The role of VDR signaling in genomic responses to 1,25(OH)<sub>2</sub>D has been studied in a mouse mammary tumor model of TNBC [164]. Cells derived from DMBA-induced tumors generated in wild-type and VDRKO mice were analyzed 24 h after treatment with 100 nM 1,25(OH)<sub>2</sub>D. As expected, genomic profiling demonstrated that 1,25(OH)<sub>2</sub>D failed to alter gene expression in VDRKO cells whereas major changes were observed in cells derived from wild-type mice (WT cell line). This study also included three clonal lines of VDRKO cells engineered to stably express human VDR (KO<sup>hVDR</sup> cells) which conferred sensitivity to the growth inhibitory effects of 1,25(OH)<sub>2</sub>D. With a twofold cutoff, 117 transcripts in WT cells and 197 transcripts in KO<sup>hVDR</sup> clones were significantly altered by 1,25(OH)<sub>2</sub>D with 35 genes found to be commonly regulated in all VDR+ cell lines (the complete list of genes is included in the manuscript). In addition to *Cyp24a1*, seven genes were validated as 1,25(OH)<sub>2</sub>D responsive and VDR dependent in this system: *Cib2*, *Prep1*, *Enpp1*, *Plau*, *Hbegf*, *Postn*, and *Has2*. The last four of these, whose expression was markedly down-regulated by 1,25(OH)<sub>2</sub>D, are known to drive breast cancer invasion and metastasis. These data support a working model whereby 1,25(OH)<sub>2</sub>D coordinately suppresses multiple genes and pathways that are required for the survival and invasion of TNBC cells.

#### 5.4 Profiling of VDR-regulated gene expression in human breast cancers

Two approaches have been used to identify potential VDR targets in human breast cancers: exposure of patient-derived tumor explants to 1,25(OH)<sub>2</sub>D ex vivo, and interrogation of publicly accessible datasets. Genome profiling in freshly isolated breast tumor explants assesses whether the vitamin D pathway is functional in the complex multicellular tumor environment that exists in vivo. In the first study of this kind, tumor slices from postmenopausal patients with stage I, II, or III breast cancer were cultured with 0.5 nM or 100 nM 1,25(OH)<sub>2</sub>D for 24h [155]. At the lower dose of 0.5 nM, 10 genes were significantly altered: *CYP24A1*, *DPP4*, *CYP26B1*, *SPIN3*, *KCKN3*, *EFTUD1*, *TKTL1*, and *CA2* (up-regulated) and *FCGR2C* and *SAMSN1* (down-regulated). Of these, *CYP24A1* was induced over sevenfold and was validated in another set of tumor samples, clearly indicating activation of VDR signaling. At 100 nM 1,25(OH)<sub>2</sub>D, 30 genes (28 up/2 down) were significantly altered by 1,25(OH)<sub>2</sub>D. In addition to those regulated by 0.5 nM 1,25(OH)<sub>2</sub>D, genes up-regulated threefold or more after treatment with 100 nM 1,25(OH)<sub>2</sub>D included *IL1RL1*, *CILP*, *P115*, *TMEM37*,

and *SHE*. The two top-down-regulated genes (2-fold or more) were *P2RY1* and *BCOR*. This dataset is publicly available on the Gene Expression Omnibus website (accession #GSE27220). The significance of this study is that it demonstrated for the first time that 1,25(OH)<sub>2</sub>D could directly influence gene expression in patient-derived multicellular tumor tissue, confirming the functionality of the VDR in human breast cancer. Although patient-to-patient variability was considerable, a clear dose–response in gene expression to the two concentrations of 1,25(OH)<sub>2</sub>D was detected. To assess whether genes altered in tumor tissue by 1,25(OH)<sub>2</sub>D were also altered in cell lines, Simmons et al. [159] integrated the dataset of breast tumor explants exposed to 100 nM 1,25(OH)<sub>2</sub>D with those from the hTERT-HME1, MCF-7 and SKBR3 cell lines. This analysis identified four genes (*CYP24A1*, *CLMN*, *EFTUD1*, and *SERPINB1*) that were up-regulated by 1,25(OH)<sub>2</sub>D in the tumor explants and in all three breast-derived cell lines. A fifth gene, *IL1RL1*, was common to all four datasets but was down-regulated in SKBR3 cells and up-regulated in the other three model systems. There were no genes identified that were down-regulated by 1,25(OH)<sub>2</sub>D in all model systems.

In a similar ex vivo explant model, genomic responses to 1,25(OH)<sub>2</sub>D were compared in explants of breast tumors and normal adjacent tissue [157,158]. This analysis demonstrated >500 differentially expressed genes in breast cancer versus 127 in normal breast tissues. Pathway analysis revealed that 1,25(OH)<sub>2</sub>D down-regulated cellular metabolic pathways and enriched pathways involved with intercellular adhesion. Remarkably, the same four genes (*CYP24A1*, *CLMN*, *SERPINB1*, *EFTUD1*) identified as common VDR targets in the analysis by Simmons et al. [159] were the most highly induced genes in this second explant study. These four genes, as well as *KLK6*—a gene that was highly induced in both tumor explants and MCF-7 cells [159]—were confirmed as 1,25(OH)<sub>2</sub>D regulated in breast cancer cell lines and in a subset of human clinical samples from normal tissue and breast cancer.

Using an unbiased genomic profiling approach, Li et al. [105] interrogated four independent datasets comprising 581 breast cancer patients with ER+ breast tumors that had been treated with tamoxifen. Stratification by tumor VDR expression in these cohorts identified biological functions enriched in VDR “high” tumors including cell death and survival, cellular proliferation, and cancer. Additional analysis indicated repression of an autophagy gene signature in VDR “high” tumors, and repression of autophagy by 1,25(OH)<sub>2</sub>D was confirmed in ER+ breast cancer cell models in vitro.

## 5.5 Summary of genomic profiling data

Genomic effects of  $1,25(\text{OH})_2\text{D}$  in breast cancer model systems have clearly been established, and the initial integration of existing datasets has identified a common set of genes that may represent biomarkers of vitamin D action for future studies. While *CYP24A1* is commonly identified in microarray studies as the most highly up-regulated gene in  $1,25(\text{OH})_2\text{D}$  treated cells, other modulated genes vary greatly depending on the model system. While not wholly unexpected as transcriptional responses across cell types are in general highly diverse, determining the molecular basis for these differences is warranted. The distinct biological effects of  $1,25(\text{OH})_2\text{D}$  in different cell types could reflect differences in the expression of VDR, its RXR partners or transcriptional co-regulators as well as the underlying epigenetic states in specific genomic regions. A caveat to the current data is that most profiles were conducted after 24 h treatment, and thus will include both primary VDR target genes and downstream effectors (i.e., secondary targets). Similarly, genes differentially expressed in tumors with high versus low VDR expression would represent multiple downstream pathways potentially regulated by vitamin D signaling. It is anticipated that alterations in these downstream targets would be more variable in different model systems. In addition, an adaptation of cells to culture, the use of long-established cell lines, and the presence of cancer-associated genomic alterations likely contribute to this diversity. However, despite the potential artifacts of cell and tissue culture model systems, it is worth noting that several of the genes identified as  $1,25(\text{OH})_2\text{D}$ -modulated in nontumorigenic mammary epithelial cells [159], including *CD14*, *CD97*, *THBD*, *NINJ1*, *CAMP*, and *IL8*, have been identified as direct VDR targets by ChIP-seq and were altered in peripheral blood mononuclear cells by oral vitamin D supplementation, but only in a subset of trial participants [165]. This supports the feasibility of identifying biomarkers of vitamin D exposure through cell culture studies but also confirms the heterogeneity of individual responses. Continued integration of the datasets reported here with additional genomic profiles and ChIP-Seq data will assist in identifying more comprehensive common and tissue/cell-specific signatures of  $1,25(\text{OH})_2\text{D}$ -VDR signaling. The continued use of complex models such as tumor explants for vitamin D studies is desirable given the expression of VDR in most cell types and the critical interactions between tumor cells and their stromal microenvironment. Furthermore, targeted analysis of vitamin D pathway expression in large publicly available datasets to address specific hypotheses or evaluate specific patient populations remains worthwhile.

## 6. Mechanistic insight into vitamin D actions in breast cancer

### 6.1 Overview

Given the large number of genes and pathways regulated by  $1,25(\text{OH})_2\text{D}$  in breast cells, it is not surprising that vitamin D inhibits cancer development and progression via multiple mechanisms. The diverse mechanisms by which vitamin D signaling alters cell and tumor behavior have been reviewed [104,143,166,167] and are summarized in Table 88.3. This section will provide a brief summary of existing data while highlighting newly recognized effects of  $1,25(\text{OH})_2\text{D}$  on cellular metabolism and cancer stem cells.

Both  $25(\text{OH})\text{D}$  and  $1\alpha,25(\text{OH})_2\text{D}$  exert profound effects on nontumorigenic VDR+ mammary epithelial cells including regulation of basal and hormone-stimulated proliferation, induction of differentiation biomarkers such as E-cadherin, regulation of apoptosis and autophagy, protection against oxidative stress and inhibition of chemical carcinogenesis [72,82,88,89,98,185]. Other potential mechanisms of vitamin D action include reduction in DNA damage (possibly via up-regulation of p53 signaling), inhibition of angiogenesis, and increased antiinflammatory signaling although these effects have primarily been demonstrated in tissues or cell types other than mammary gland [185–198]. The possibility that  $1,25(\text{OH})_2\text{D}$  modulates cross-talk between mammary epithelial cells and immune responses is supported by the identification of numerous cytokines and their receptors as VDR targets including *CD14*, *IL1RL1*, and *IL1 $\alpha$*  [159,199,200]. In established breast cancer cells,  $1,25(\text{OH})_2\text{D}$  modulates key cell cycle regulators to induce cell cycle arrest, trigger differentiation, and/or activate cell death via apoptosis or autophagy. As discussed above, cells derived from VDRKO mice have been used to definitely establish that VDR is required for the antiproliferative and pro-apoptotic effects of  $1,25(\text{OH})_2\text{D}$  in vitro and in vivo [133,201,202].

Interactions between VDR and estrogen signaling contribute to the effects of  $1,25(\text{OH})_2\text{D}$  in hormone-dependent breast cancer cells. The  $1,25(\text{OH})_2\text{D}$ -VDR complex directly represses ER via two negative vitamin D response elements and also represses estrogen synthesis via inhibition of aromatase transcription [203,204]. These combined actions lead to the disruption of estrogen-stimulated proliferation and survival pathways in vitro and in an in vivo model of hormone-responsive breast cancer associated with obesity [138]. Given that the majority of human breast cancers are hormone-dependent and that ER+ tumors have the highest VDR expression, optimizing vitamin D status to abrogate estrogen-driven pathways in breast tumors

**TABLE 88.3** In vitro effects of vitamin D pathway in breast cancer model systems.

Model systems	Effects of VDR agonists <sup>a</sup>	Selected Refs.
<b>Proliferation</b>		
MCF-7 MDA-MB-231 Hs578T WT & VDRKO	Cell cycle arrest ↓ cyclin D1, ↓ Ki67 marker of proliferation ↑ p27/p21 ↓ Wnt/β-catenin signaling ↓ HAS2 expression and HA secretion	[168–170]
<b>Apoptosis</b>		
MCF-7 MDA-MB-231 MDA-MB-453	Induction of apoptosis ↑ Bax expression, ↓ Bcl-2 expression Caspase activation ↓ RAS, MER, ERK1/2 expression ↓ mTOR expression & ↑ AMPK activation	[171–174]
<b>Autophagy</b>		
Normal mammary gland MCF-7 LCC9 (tamoxifen resistant MCF7 clone)	↑ Beclin 1, LC3-I, LC3-II expression ↓ Bcl-2 expression Inhibit tamoxifen driven survival autophagy via reversal of unfolded protein response	[82,105,175]
<b>Oxidative stress</b>		
MCF-7 MDA-MB-231	Disrupt cellular iron homeostasis Induce oxidative stress, cell death : ↓ ROS level, γ-H2AX level catalase expression	[176]
<b>Stemness phenotype</b>		
SUM159 MCF10DCIS MCF-7 BT474	Reduce cancer stem cell populations ↓ tumorsphere forming efficiency ↓ cancer stem cell markers (ALDH, CD44, OCT4, notch1/2/3, JAG1/2, NFκB) ↓ GDF15 expression ↓ Wnt/β-catenin signaling	[177–179]
<b>EMT and metastasis</b>		
MDA-MB-231 MCF-7 HME model of EMT	Reduce migration & EMT markers ↑ E-cadherin/ ↓ vimentin Enhance cellular stiffness Down-regulation of VDR expression during EMT	[111,174,180,181]
<b>Cellular metabolism</b>		
MCF-7, MDA-MB-231 hTERT-HME1 cells, MCF10A and HME transformation models	<i>Metabolic effects of 1,25(OH)2D are cell type dependent</i> In nontransformed HME cells: ↓ glutamine synthetase/ ↑ glutamate transporter SLC1A1; ↓ glutamine oxidation, enhance glutathione accumulation In MCF10A transformation model: ↓ glycolytic flux, alter redox balance, ↓ glucose uptake, alter glycolytic gene expression; ↓ pyruvate carboxylase, ↓ triglycerides & palmitate synthesis, In MCF7: ↑ acidification rate and serine accumulation, ↓ cholesterol accumulation via ↑ ABCA pump In TNBC: ↓ mitochondrial respiration In both luminal and TNBC cells: ↓ V-H <sup>+</sup> -ATPase protein pump, ↑ GAPDH, enhance AMPK activity	[159,172–174,182–184]
<b>Estrogen metabolism and signaling</b>		
MCF-7 SUM149 MDA-MB-231	Regulate ERRα-ERα cross talk of estrogen signaling activation ↑ ERRα, VDR, CYP24A1 expression Re-expression of ER in TNBC cells	[170,171]

<sup>a</sup>Includes effects of natural and synthetic VDR agonists.



could translate into the prevention or delay of tumorigenesis for large numbers of women. This concept is supported by the proven action of the antiestrogen tamoxifen which was shown in RCTs to reduce breast cancer development by approximately 50% in women at high risk of breast cancer [205]. Related to this, high tumor VDR expression was associated with longer recurrence-free survival in tamoxifen-treated breast cancer patients [105].

Although estrogen-independent breast cancer cells, representative of late-stage disease, are in general less sensitive to 1,25(OH)<sub>2</sub>D-mediated cell cycle regulation, effects on genomic stability, invasion, and metastatic potential have been demonstrated. For instance, 1,25(OH)<sub>2</sub>D stabilized the 53BP1 protein and selectively eliminated invasive breast cancer cells expressing mutant *BRCA1* [206]. 1,25(OH)<sub>2</sub>D consistently inhibits invasion as measured in Boyden chambers [123] and metastasis as measured in 3D culture models [180]. Potential mechanisms by which vitamin D signaling reduces invasion and metastasis include suppression of extracellular proteases (MMP-9, urokinase-type plasminogen activator, tissue-type plasminogen activator), protease inhibitors, and adhesion molecules [164,207]. 1,25(OH)<sub>2</sub>D also inhibits the synthesis and secretion of hyaluronic acid, a major component of the tumor microenvironment that drives progression [168,208].

Many recent studies have explored whether vitamin D signaling exerts additive or synergistic effects with other agents. Thakkar et al. [209] characterized 15 breast cancer cell lines and reported that approximately two-thirds of TNBC cell lines co-express AR and VDR and that simultaneously targeting these two receptors resulted in additive antiproliferative effects. Other combination therapies that have recently been shown to exert additive or synergistic effects with VDR agonists in breast cancer cells include metformin [210], cox-2 inhibitors [211], the EGFR inhibitor gefitinib [212], ionizing radiation [213], the multikinase inhibitor dovitinib [214] and many others. A detailed discussion of these many studies is beyond the scope of this chapter, and a recent review is available on this topic [215].

## 6.2 Emerging role of vitamin D pathway in cancer cell metabolism

A role for vitamin D in the regulation of energy and amino acid metabolism of both normal and tumor cells has been suggested based on genomic and metabolomic analyses [157,159,216]. This role is of considerable interest given the adaptive changes in energy metabolism required for rapid proliferation and macromolecule synthesis in cancer cells [217,218]. The “metabolic switch” originally proposed by Warburg [217] is now known to

be promoted by oncogenes such as *MYC* and *RAS* and prevented by tumor suppressors such as *TP53* [219–225]. Agents that block *MYC* activation and/or elicit p53-like actions to disrupt the metabolic adaptations in glucose and amino acid metabolism and abrogate the metabolic switch would theoretically have therapeutic potential.

In this context, 1,25(OH)<sub>2</sub>D has been shown to regulate glucose and amino acid metabolism in association with growth inhibition of nontumorigenic mammary epithelial cells [182,216,226]. The glutamate transporter EAAT3 (encoded by *SLC1A1*) and enzyme glutamine synthetase (GS, encoded by *GLUL*) were identified and confirmed as 1,25(OH)<sub>2</sub>D targets in hTERT-HME1 cells [159,161,182]. 1,25(OH)<sub>2</sub>D increased *SLC1A1* mRNA (>10-fold) and decreased *GLUL* mRNA (>4-fold) with predicted effects on expression of the cognate proteins EAAT3 and GS. In 1,25(OH)<sub>2</sub>D treated cells, changes in expression of *SLC1A1* and several other glutamate transporters correlated with altered cellular glutamate handling, accumulation of glutathione, and changes in respiratory capacity. Suppression of GS by 1,25(OH)<sub>2</sub>D is particularly intriguing as this enzyme is required for the conversion of glutamate to glutamine, a nitrogen donor essential for production of purine and pyrimidine nucleotides during cell proliferation [218]. Indeed, 1,25(OH)<sub>2</sub>D treated cells exhibited reduced mitochondrial respiration of glutamine and failed to adapt to glutamine deprivation. Furthermore, exogenous glutamine partially rescued growth inhibition by 1,25(OH)<sub>2</sub>D. These findings demonstrating regulation of *SLC1A1* and *GLUL* by 1,25(OH)<sub>2</sub>D are intriguing because: (a) glutamate uptake and glutamate transporters are enhanced during differentiation and deregulated in cancer cells; (b) *Slc1a1* null mice exhibit GSH deficiency and high oxidative stress and (c) GS enzymatic activity is necessary for adaption of mammary cells to glutamine depletion [227–230]. Furthermore, data on The Human Protein Atlas indicate that *SLC1A1* is reduced and GS is increased in human breast cancers relative to normal tissue. These studies support the concept that regulation of *SLC1A1* and *GLUL* by 1,25(OH)<sub>2</sub>D may synergize with p53 to alter metabolic flux, prevent the metabolic switch and induce quiescence in normal mammary epithelial cells.

Consistent with the regulation of amino acid transporters by 1,25(OH)<sub>2</sub>D in hTERT-HME1 cells, Zhou et al. [226] reported that 1,25(OH)<sub>2</sub>D repressed the *SLC1A5* glutamine transporter in RAS-transformed MCF10A (MCF10A-ras) cells and identified a negative VDRE site in the proximal promoter of this gene. *SLC1A5* encodes a major plasma membrane glutamine transporter and its down-regulation by 1,25(OH)<sub>2</sub>D was associated with reduced intracellular glutamine and glutamate concentrations. This group also

compared the regulation of cellular glucose metabolism by  $1,25(\text{OH})_2\text{D}$  in MCF10A-ras cells and the premalignant MCF10A cells from which they were derived [216]. In this model,  $1,25(\text{OH})_2\text{D}$  reduced the flux of glucose through glycolysis in both MCF10A and MCF10A-ras cells, with more pronounced effects in the transformed cells.  $1,25(\text{OH})_2\text{D}$  also reduced the flux of glucose to acetyl-coA and oxaloacetate in both cell lines, suggesting a reduction in TCA cycle activity. The predicted consequences of the  $1,25(\text{OH})_2\text{D}$  induced changes reported in this series of studies would be limited availability of TCA-derived precursors for macromolecule synthesis coincident with a reduction in proliferation.

Comprehensive comparison of luminal (MCF-7, T47D) and TNBC (MDA-MB-231, MDA-MB-468, HCC-1143) cells revealed that  $1,25(\text{OH})_2\text{D}$  affects metabolism in a cell-type dependent fashion [183]. In luminal cells,  $1,25(\text{OH})_2\text{D}$  enhanced the acidification rate (indicative of glycolysis) and induced serine accumulation, whereas in TNBC cells the main effect was to reduce mitochondrial respiration. In all breast cancer cell lines,  $1,25(\text{OH})_2\text{D}$  induced GAPDH and AMPK signaling. Despite the potential pro-survival impact of these actions,  $1,25(\text{OH})_2\text{D}$  sensitized cells to chemotherapeutic agents including 5-fluoruracil. Continued exploration into the actions of  $1,25(\text{OH})_2\text{D}$  on glucose, glutamate, and glutamine metabolism is warranted to fully determine its role in the regulation of these critical pathways during breast cancer onset and progression.

### 6.3 Regulation of breast cancer stem cell populations by $1,25(\text{OH})_2\text{D}$

The existence of cancer stem cells (CSCs) in human and rodent breast cancers is widely supported by clinical and experimental data [231]. The CSC theory proposes that breast cancers arise from a minor subpopulation of transformed breast progenitor cells which are necessary and sufficient for the initiation and maintenance of tumors. CSCs can be identified by surface markers ( $\text{CD44}^{\text{HI}}/\text{CD24}^{\text{NEG/LOW}}$  for human;  $\text{CD49f}^{\text{HI}}/\text{EpCam}^{\text{LOW}}$  for mouse) and/or activity of aldehyde dehydrogenase 1 (ALDH1+) and can be propagated in vitro as nonadherent spheres ("mammospheres"). Verification of the stemness of CSCs and their frequency is through limiting dilution assays in which cells are injected into host mice at increasing doses and the percentage of CSCs within the original cell population is calculated based on tumor incidence. Pathways linked to survival of CSCs include Wnt, Notch, and Sonic Hedgehog, all of which play a role in breast cancer progression. With respect to cancer treatment, it is well-accepted that CSCs display remarkable resistance to conventional therapies including radiation

and chemotherapeutics. It is anticipated that nontraditional therapies able to bypass the drug resistance of CSCs leading to their selective elimination will constitute new and highly effective strategies for the treatment of human cancers.

In one of the first studies to address the impact of vitamin D signaling on CSCs, Pervin et al. [232] demonstrated significantly reduced levels of VDR protein in mammospheres isolated from SKBR3, MCF-7, and HME-Ras cells compared to cells grown on plastic. The low expression of VDR in mammospheres precluded significant effects of  $1,25(\text{OH})_2\text{D}$  and  $25(\text{OH})\text{D}$  on gene expression. SKBR3 cells sorted for ALDH+ had higher expression of CD44 and lower expression of VDR than ALDH- populations, suggesting an inverse correlation between stemness and VDR. In SKBR3 cells, overexpression of VDR reduced, whereas knockdown of VDR enhanced, mammosphere number. The importance of this study was that it demonstrated a functional role for VDR in mammospheres which were shown to be enriched in cells positive for stem cell markers CD44 and ALDH. Further follow-up on the expression and regulation of VDR in the stem cell models used in this study is warranted.

In another study of human breast cancer cell lines, Thakkar et al. [209] examined whether  $1,25(\text{OH})_2\text{D}$  altered differentiation state, CSC marker expression, or tumorsphere forming efficiency of TNBC lines MFM-223 and CAL-148. In both lines,  $1,25(\text{OH})_2\text{D}$  inhibited tumorsphere formation, decreased the percentage of ALDH+ cells, and reduced CSC-associated markers including CD44. Interestingly, both of these cell lines have high expression of AR and combination therapy with AR and VDR agonists had additive effects on decreasing the CSC population and stemness markers including CD49f, SOX2, OCT4, Musashi, and Notch.

The effects of  $1,25(\text{OH})_2\text{D}$  and its Gemini analog BXL0124 on stem cell markers were studied in MCF10DCIS.com cells, which constitute a model of early stage basal-like breast cancer. MCF10DCIS.com cells are enriched in cells with high CD44 expression and form DCIS-like lesions when xenografted into immunodeficient mice. In vivo, BXL0124 reduced the growth of MCF10DCIS grafts and decreased tumor expression of both CD44s (standard form of CD44) and the variant CD44 forms which are often expressed in tumors (CD44v). Both  $1,25(\text{OH})_2\text{D}$  and BXL0124 strongly repressed CD44 proteins in vitro in a VDR and p53-dependent manner. Follow-up studies with this model [233] demonstrated that both vitamin D compounds reduced the  $\text{CD44}^{\text{HI}}/\text{CD24}^{\text{NEG/LOW}}$  population, decreased mammosphere forming efficiency, and repressed stemness and pluripotency markers including CD44, CD49f, c-Notch1, pNFkB, OCT4, and KLF-4. Mechanistically, the  $\text{CD44}^{\text{HI}}/\text{CD24}^{\text{NEG/LOW}}$  population

was found to be enriched in Notch activity and the amount of active Notch receptor was decreased by BXL0124, leading to reduction of downstream mediators including MYC. These effects were linked to the induction of HES-1 (an inhibitor of Notch1 signaling) by BXL0124 [234], supporting a model whereby BXL0124 suppresses the CD44<sup>HI</sup>/CD24<sup>NEG/LOW</sup> population of CSCs by HES1-mediated inhibition of Notch signaling.

Recent comprehensive genomic analysis of 1,25(OH)<sub>2</sub>D treated tumorspheres [177] reported 439 genes with >4-fold positive change and 703 genes with >4-fold negative change in normalized RNA expression compared to control tumorspheres. Both 1,25(OH)<sub>2</sub>D and BXL0124 down-regulated genes involved in the maintenance of breast cancer stem-like cells (e.g., *GDF15*), epithelial-mesenchymal transition, invasion and metastasis (e.g., *LCN2*, *S100A4*) and chemoresistance (e.g., *NGFR*, *PPP1R1B*, *AGR2*) while up-regulating genes associated with a basal-like phenotype (e.g., *KRT6A*, *KRT5*) and negative regulators of breast tumorigenesis (e.g., *EMP1*). Through comparison of their RNA-Seq data with published databases of human mammary gland and breast cancer cell lines, the group reported *TP63*, *CYP24A1*, *CD14*, *TRPV6*, *STAT4*, and *FABP6* as genes commonly regulated by 1,25(OH)<sub>2</sub>D and BXL0124. In particular, the p63 protein (an inducer of myoepithelial differentiation whose loss promotes migration, invasion, and distant metastasis) was validated as a VDR target in tumorspheres. Collectively, these data support the concept that vitamin D signaling prevents DCIS progression to invasive ductal carcinoma by inducing myoepithelial differentiation of the cancer stem-like cells. This concept is consistent with the reduced incidence of human DCIS with vitamin D supplementation as noted earlier [21,49].

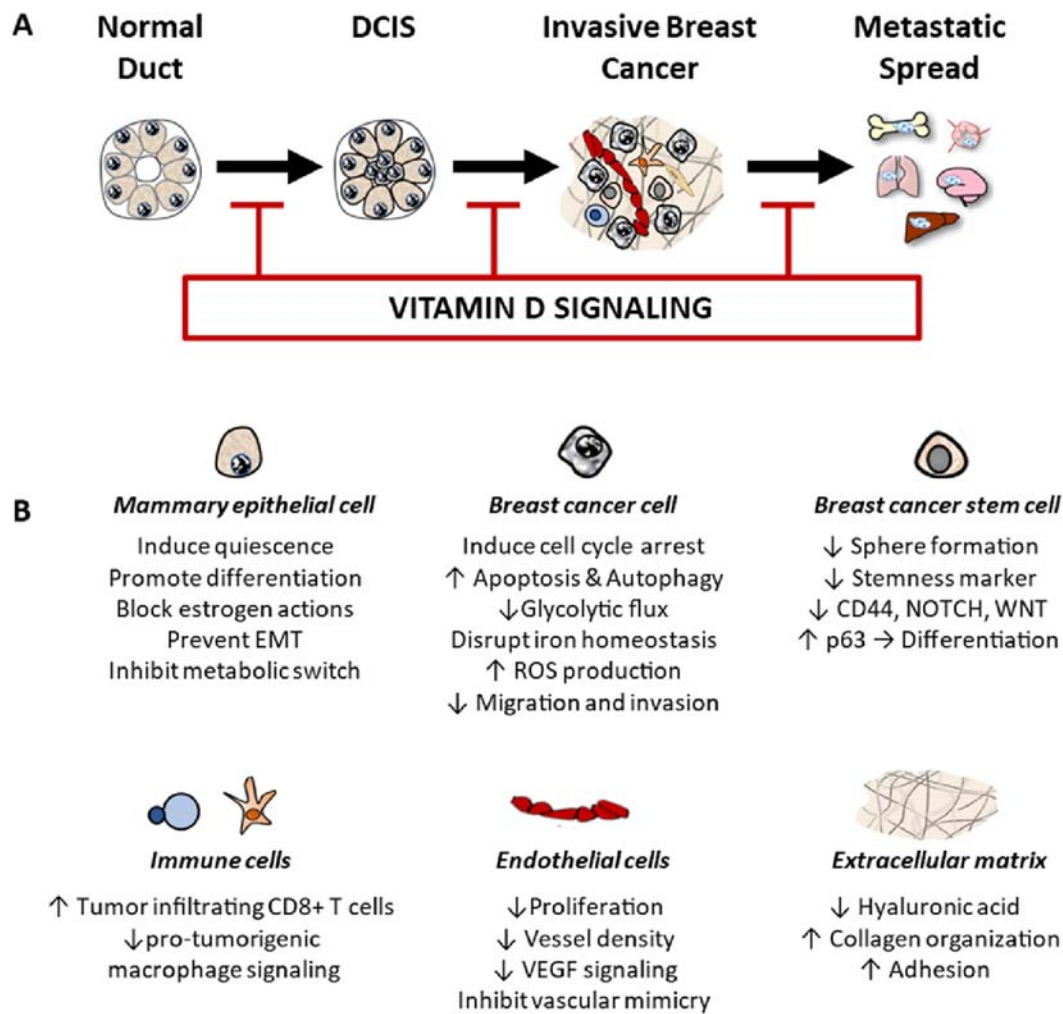
Similar inhibitory effects of 1,25(OH)<sub>2</sub>D on stem cell populations have been demonstrated in a *Wnt1*-driven murine mammary tumor model [139]. The authors first confirmed VDR pathway function in CSCs (CD49f<sup>HI</sup>/EpCam<sup>LOW</sup>) sorted from *Wnt1* tumorgrafts. Secondly, they demonstrated that 1,25(OH)<sub>2</sub>D at concentrations as low as 0.1 nM inhibited spheroid formation in primary culture and self-renewal in secondary passages. Most importantly, in vivo limiting dilution assays demonstrated a significant reduction in the frequency of CSCs in *Wnt1* cultures pre-treated with 1 nM 1,25(OH)<sub>2</sub>D. Gene expression assays on CSCs showed that 1,25(OH)<sub>2</sub>D reduced the expression of multiple WNT pathway genes including *Lef1*, *Axin2*, and *Ccnd*

and that forced expression of  $\beta$ -catenin abrogated the effects of 1,25(OH)<sub>2</sub>D on spheroid formation. These data extend the known targets of 1,25(OH)<sub>2</sub>D in CSCs to the WNT pathway, another critical driver of stemness and pluripotency.

In summary, these studies highlight the existence of a functional vitamin D pathway in CSCs which acts to robustly alter gene expression and inhibit stem cell function. While the clinical relevance of these observations remains to be seen, the work adds new insight into the actions of vitamin D in breast cancer and provides fertile ground for additional research. It is worth noting that the surface markers used to identify CSCs also function to mediate cell-cell interactions and activate survival pathways. In particular, CD44 is a cell surface glycoprotein that drives STAT3 survival signaling when activated by its ligand hyaluronic acid, the accumulation of which has consistently been associated with poor prognosis in breast cancer [235]. The hyaluronic acid pathway is also targeted by VDR in aggressive breast cancer cells [164], as 1,25(OH)<sub>2</sub>D markedly suppresses hyaluronan synthase-2 (HAS2) expression and decreases hyaluronic acid secretion. These effects of 1,25(OH)<sub>2</sub>D would be expected to further compromise CSC viability via the interruption of CD44-STAT3 survival signaling.

#### 6.4 Vitamin D pathway in tumor-infiltrating cells and extracellular matrix

In addition to the cancerous cells, many nonepithelial cell types accumulate in tumors including stromal fibroblasts, adipocytes, immune cells, and vascular endothelial cells. Increasing evidence suggests that the nonepithelial cells, which together with the extracellular matrix make up the tumor microenvironment, contribute to disease progression. It is worth noting that most of these cell types express VDR, creating a complex network of interacting cells that respond to 1,25(OH)<sub>2</sub>D (Fig. 88.2). With respect to breast cancer, VDR signaling has been shown to block pro-tumorigenic signaling from tumor-associated macrophages [96], inhibit endothelial cell proliferation and angiogenesis [214,236,237], alter extracellular matrix composition and components including collagen and hyaluronic acid [78,168,238], alter cytokine secretion from adipocytes [79] and increase the infiltration and activity of antitumor lymphocytes [142]. For general discussion of vitamin D and the tumor microenvironment, please see Wu et al. [239].



**FIGURE 88.2** Summary of vitamin D actions during breast cancer development and progression.

## 7. Conclusion

Despite significant progress in understanding the relationships between vitamin D and breast cancer, many issues remain unresolved. Much of the work conducted in cell systems or animal models are consistent, but epidemiological data remains mixed and clinical studies are limited. As discussed in this chapter, population studies largely support the concept that high serum levels of 25(OH)D are associated with lower risk of initial disease development and may retard progression. However, questions remain regarding mechanisms, tissue specificity, and optimal intake/blood concentration of vitamin D for potential benefits on cancer. Tissue uptake and cellular metabolism of vitamin D metabolites in vivo are likely to be highly relevant to cancer biology, yet few studies have successfully measured these parameters. With respect to breast cancer in particular, the microenvironment surrounding the epithelial cells at risk for carcinogenesis clearly contributes to risk,

and important functions of the VDR in adipocytes, stromal cells, immune cells, and vascular networks have been identified. Given the strong association of breast cancer and obesity, a renewed focus on vitamin D storage, metabolism, and actions in breast adipose tissue is warranted. With the emerging promise of immune therapies for solid cancers, efforts to dissect VDR actions with respect to immune surveillance in the context of breast cancer are also of high interest.

Further mechanistic insight into potential interaction between systemic vitamin D status and other known breast cancer risk factors including genetic (*BRCA1*, *BRCA2*, *ATM*), endocrine (estrogen, progesterone), and environmental (radiation, carcinogens) modulators is also needed. Genomic profiling has characterized many 1,25(OH)<sub>2</sub>D responsive targets in normal mammary cells and in breast cancers providing valuable insight into the molecular actions of 1,25(OH)<sub>2</sub>D and the VDR in regulation of cell cycle, apoptosis, and differentiation as well as regulation of tumor metabolism and



inhibition of cancer stem cells. As noted above, the role of VDR in individual cell types (i.e., epithelial, adipose, fibroblast, endothelial, immune) of normal and tumor tissues remains to be clarified. Furthermore, there has been limited attention directed at understanding how VDR integrates signaling between these diverse cell types and what soluble signals and paracrine pathways may be regulated by  $1,25(\text{OH})_2\text{D}$  in the tissue and tumor microenvironment. Finally, the possible interactions of VDR with other nuclear receptors (such as AR, ER, PGR, and RXRs) and their ligands in control of mammary cell fate/carcinogenesis are worthy of additional study. Comprehensive genomic, metabolomics, and proteomic profiling approaches combined with mechanistic studies remain highly valuable for the identification of relevant biomarkers of tissue vitamin D action that are needed to guide translational investigations such as supplementation trials.

## 8. Summary points

- VDR and the vitamin D metabolic enzymes CYP27B1 and CYP24A1 are expressed in the normal mammary gland and in the majority of human breast tumors.  $1,25(\text{OH})_2\text{D}$  and other VDR agonists exert tumor suppressive effects in breast cancer model systems both in vitro and in vivo. These effects are mediated via profound changes in the expression of genes associated with tumor-suppressive pathways.
- Population studies have demonstrated associations between vitamin D status (as measured by serum  $25(\text{OH})\text{D}$ ) and breast cancer risk and outcome. The association of higher vitamin D status with delayed recurrence and improved survival is convincing.
- In supplementation trials, vitamin D reduced the development of DCIS, a preneoplastic lesion, and was associated with a reduced risk of advanced/metastatic cancers.
- Vitamin D deficiency is common in breast cancer patients, particularly those with aggressive disease. Correction of vitamin D deficiency has the potential to improve outcomes in women living with breast cancer.

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## Vitamin D and colorectal cancer

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### OBJECTIVES

- Present the effects of  $1,25(\text{OH})_2\text{D}_3$  on cultured colon carcinoma cells, including the multifaceted antagonism of the Wnt/ $\beta$ -catenin signaling pathway.
- Present the effects of  $1,25(\text{OH})_2\text{D}_3$  on CRC patient-derived stromal tumor fibroblasts and colorectal organoids.
- Present and discuss the indirect actions of  $1,25(\text{OH})_2\text{D}_3$  that may affect CRC by modulating angiogenesis, immune response, detoxification, and microbiome.
- Review the literature on the effects of vitamin D,  $1,25(\text{OH})_2\text{D}_3$  or analogs in animal models of CRC.
- Discuss the discrepancy between the results of observational and experimental studies supporting a protective action of vitamin D against CRC and the unclear data from human intervention studies.

mutations by the genetic background, environment (mainly lifestyle factors and diet), ontogeny, and anatomy. Thus, there are differences in the mutational landscape and the clinical development, management, and response to therapies for right/ascending colon, left/descending colon, and rectal tumors. Sporadic CRC was for long considered a disease mostly diagnosed in the fifth or sixth decade of life or later; however, its incidence is increasing in people before age of 50 (early-onset CRC or EOCRC) while decreasing in the older population [1]. EOCRC shows specific genetic, epigenetic, and clinical features [2,3]. CRC heterogeneity is multifaceted: its incidence is higher among men, but women have a higher incidence of right-sided CRC, while EOCRC mortality is higher for men versus women and its incidence is higher among people of color versus white people particularly in right-sided colon tumors [4]. Two additional factors that modify the risk of CRC are intestinal microbiota and chronic inflammation, as shown by the increased risk of CRC in patients with inflammatory bowel disease (IBD: ulcerative colitis (UC) and Crohn's disease (CD)) [5,6].

### 1. Introduction

#### 1.1 Colorectal cancer

Colon/colorectal cancer (CRC) is a group of diseases that result from the malignant transformation of the epithelium lining the external surface of the large intestine. CRC is one of the most frequent neoplasia and a leading cause of death worldwide. CRC is heterogeneous in relation to age, location, alterations, sex, and race, which probably reflects the modulation of acquired

#### 1.2 Genetics and subtypes of colorectal cancer

CRC is the best characterized solid neoplasia in terms of genetic alterations. The malignant transformation of the intestinal epithelial cells occurs because of mutations and epigenetic modifications that are thought to progressively accumulate over 10–20 years. Due to this estimated long period of development, CRC represents an excellent model to interrogate the potential interactions between genetic alterations and environmental modifiable risk factors affecting cell epigenetics and thus,

gene expression and phenotype, identified as contributing to the disease.

In 1990, Fearon and Vogelstein proposed the first genetic model for CRC based on the accumulation of alterations in tumor suppressor genes (*adenomatous polyposis coli* or *APC*, *TP53*) and oncogenes (*KRAS*), known today as the suppressor pathway that leads to chromosomal instability and most (nonhypermuted) CRC cases [7]. Later, a second, alternative pathway, the mutator pathway, was proposed that affects a low proportion of cases (5%–10%), implies the loss or mutation of genes (*MSH2*, *MLH1*, *MSH6*, *PMS2*) that are involved in the repair of DNA replication errors or a 3' deletion in the *EPCAM* gene that causes the epigenetic silencing of *MSH2*. This mutator pathway is responsible for the microsatellite instability (MSI) or replication error phenotype causing the progressive accumulation of thousands of small mutations (DNA instability) characteristic of Lynch syndrome/hereditary nonpolyposis colon cancer (HNPCC) [8]. MSI also appears in sporadic CRC as a result of gene mutation or silencing (*MLH1*) in adult life, globally representing 15%–20% of total CRC. Another cause of hypermutated CRC is the alteration of *POLE* or *POLD1* genes encoding DNA polymerases involved in DNA base substitution. For an update on CRC genetics (germline and somatic mutations, epigenetic silencing, familial and sporadic, polyposis and nonpolyposis syndromes, and EOCRC diagnosed at young age), we recommend some reviews [9,10].

In addition to these stepwise models, a “Big Bang” model of single genomic catastrophe event and a “Cancer Punctuated Equilibrium” model of synchronous multiple mutagenesis episodes alternating with stable periods (stasis) have also been proposed as promoting CRC progression [11,12]. The majority of CRC cases are sporadic; only a minor (5%–10%) proportion of CRC is hereditary and includes several syndromes such as familial adenomatous polyposis (FAP), HNPCC, *MUTYH*-associated polyposis, Peutz-Jeghers syndrome, and *PTEN*-hamartomatous tumor syndrome. These syndromes are caused by the inheritance of mutated alleles of high penetrance, major tumor suppressor genes (*APC*; FAP), or DNA repair genes (mainly *MSH2* or *MLH1*; HNPCC). Additionally, around one-fourth of cases have a familial component whose genetic basis is mostly unknown and may be due to the inheritance of “negative” alleles of genes of moderate penetrance. These alleles may have little effect individually but contribute significantly to increasing CRC risk when they accumulate in specific combinations.

Molecular subtypes of CRC with partially different alterations, biological features, and clinical behavior have been proposed. Although its value predicting patient survival has been questioned [13], a classification of

CRC in four consensus molecular subtypes (CMS) is widely accepted: CMS1, with MSI and strong immune activation; CMS2, with marked Wnt/ $\beta$ -catenin pathway activation; CMS3, with epithelial and metabolic dysregulation; and CMS4, with strong transforming growth factor (TGF)- $\beta$  activation and stromal invasion [14]. Notably, the analysis of the gene expression profiles associated with stem-like/mesenchymal, poor-prognosis CMS4 CRC has revealed that most upregulated genes are induced by TGF- $\beta$  and expressed by stromal cells: fibroblasts (cancer-associated fibroblasts or CAFs, a heterogeneous population of cells of diverse origin), leukocytes, or endothelial cells [15,16]. These CMS distribute unevenly along colon segments and rectum and may contribute to differences in clinicopathological features, patterns of metastases, clinical development and chemosensitivity for right/ascending colon, left/descending colon, and rectal tumors [17–20]. In the healthy intestine, epithelial cells and fibroblasts exchange signals in a dynamic cross-talk that ensures tissue homeostasis, which is lost in tumors. Carcinoma cells secrete factors that alter (“activate”) the phenotype of normal fibroblasts toward CAFs, which in turn change that of carcinoma cells by secreting factors that act directly on them or that modulate other stromal cells (endothelial, pericytes, immune cells) and the extracellular matrix (ECM). In line with this, CAFs secrete exosomes that promote epithelial-to-mesenchymal transition (EMT), cell stemness, metastasis, and chemotherapy resistance in CRC, and together with immunosuppressive and tumor-promoting M2 macrophage markers can predict outcome in CRC patients [21–23]. Although CAFs have been for long considered as genetically stable, work in mice models has revealed that alterations in intestinal fibroblasts can contribute to the tumorigenic process [24]. Recent data suggest that the protumorigenic effect of CAFs acting on carcinoma cells is stronger in inflammation-associated CRC than in sporadic CRC [25]. Thus, tumor stroma, particularly CAFs, seems to play a critical role in CRC progression, which agrees with the concept that tumors are composed of genetically/epigenetically altered cancer cells and recruited phenotypically altered stromal cells that interact and mutually benefit in a dynamic fashion.

Interestingly, a comprehensive analysis of premalignant human colorectal polyps has defined two distinct paths for precancer to cancer transformation involving genetic/epigenetic alterations and differential immune microenvironment features: conventional adenomas arising from Wnt-driven expansion of crypt stem cells and serrate polyps that derive from differentiated cells through metaplasia [26].

Most CRC display alterations in proto-oncogenes and tumor suppressor genes, leading to the deregulation of a few signaling pathways: Wnt/ $\beta$ -catenin, EGFR-RAS-

RAF, PI3K, p53, and TGF- $\beta$ -SMADs [27,28]. Two findings emphasize the crucial importance of the Wnt/ $\beta$ -catenin pathway in CRC. First, massive sequencing efforts have shown that over 94% of primary and up to 96% of metastatic colorectal tumors, either hypermutated or nonhypermutated, contain mutations in genes that aberrantly activate the Wnt/ $\beta$ -catenin signaling pathway [27,28]. Second, it has been reported that an intestinal stem cell gene signature that largely coincides with that induced by the activation of the Wnt/ $\beta$ -catenin pathway identifies CRC stem cells and predicts disease relapse [29].

### 1.3 The Wnt/ $\beta$ -catenin signaling pathway

Wnt signaling is fundamental for the development of multicellular organisms regulating key processes such as primary axis formation, asymmetrical stem cell division, organogenesis, and regeneration [30,31]. In the adult, Wnt signaling plays an essential role in tissue homeostasis, and its deregulation is frequently associated with cancer initiation and/or progression [32,33]. CRC is the most paradigmatic example of Wnt signaling deregulation.

The Wnt family of extracellular ligands is composed of 19 members in mammals. Wnts are secreted glycoproteins, hydrophobic due to lipid modification and of compact structure because of abundant disulfide bonds [34]. There are a great variety of Wnt receptors and coreceptors, although the bases for ligand–receptor binding specificity are mostly unknown [35]. Nevertheless, this abundance of receptors allows Wnt proteins to engage different signaling pathways [31,36]. In this chapter, we will focus on the Wnt/ $\beta$ -catenin signaling pathway or Wnt canonical pathway, which is well studied and its involvement in cancer, in particular CRC, is well documented. Other Wnt pathways are globally known as Wnt noncanonical pathways and their role in tumorigenesis is less clear, although some evidence is beginning to mount [37–39].

In the canonical pathway, Wnt proteins control the amount of free cytosolic  $\beta$ -catenin in target cells. In unstimulated epithelial cells,  $\beta$ -catenin is mostly located at plasma membrane *adherens junctions* bound to the cytoplasmic tail of the  $\text{Ca}^{2+}$ -dependent intercellular adhesion molecule E-cadherin. Free  $\beta$ -catenin in the cytoplasm is targeted for proteolysis by a multiprotein destruction complex that contains the tumor suppressor proteins APC and Axin, and the serine/threonine kinases casein kinase (CK) $\text{I}\alpha$  and glycogen synthase kinase (GSK) $3\text{-}\alpha/\beta$ . N-terminal phosphorylation within this complex primes  $\beta$ -catenin for subsequent ubiquitination by  $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP) and proteasomal degradation. Therefore, in

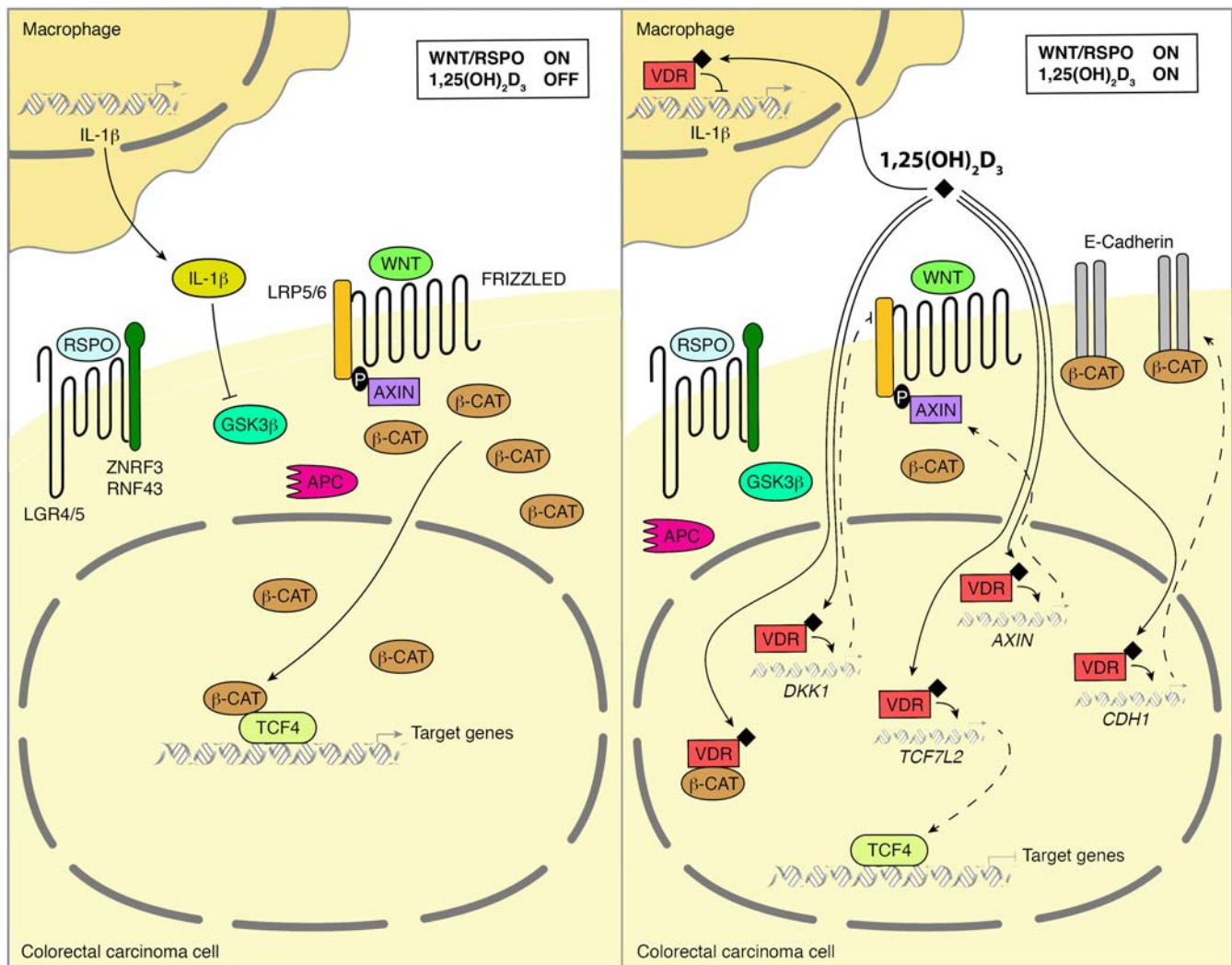
the absence of Wnt ligands, levels of free  $\beta$ -catenin are kept low [31,36] (Fig. 89.1).

The canonical pathway is triggered upon Wnt binding to a heterodimeric cell surface receptor composed of a member of the Frizzled family of seven-pass transmembrane receptors and LRP5 or 6, members of the LDL receptor-related protein (LRP) family. Wnt binding induces LRP5/6 phosphorylation and its subsequent interaction with Axin, which results in inactivation of the  $\beta$ -catenin destruction complex and, therefore, the accumulation of  $\beta$ -catenin in the cytoplasm [31,36]. A proportion of  $\beta$ -catenin then enters the nucleus and interacts with transcription factors of the T cell factor (TCF) family (four members, in colon preferentially TCF4 encoded by *TCF7L2* gene). In the absence of  $\beta$ -catenin, TCF proteins and some corepressors are bound to their target genes, in most cases actively repressing them.  $\beta$ -Catenin behaves as a coactivator, displacing the corepressors and reversing the regulation of TCF targets: genes that are repressed in the absence of nuclear  $\beta$ -catenin (many involved in cell proliferation, and migration/invasion) become activated, while a minority that are activated in the absence of nuclear  $\beta$ -catenin become repressed following  $\beta$ -catenin binding to TCF [36,40,41].

The Wnt/ $\beta$ -catenin pathway is additionally regulated by transmembrane E3 ubiquitin ligases ring finger protein 43 (RNF43) and zinc and ring finger 3 (ZNRF3), which constitutively promote the degradation of Frizzled receptors causing the repression of Wnt/ $\beta$ -catenin signaling. Overcoming this effect, members of the R-spondin family of proteins (RSPO1–4) bind to leucine-rich repeat-containing G protein–coupled (LGR) receptors and inhibit RNF43/ZNRF3, which leads to Wnt/ $\beta$ -catenin signaling sustaining [42,43] (Fig. 89.1). RSPOs are produced by stromal cells adjacent to the stem cell niche [44] and are necessary to maintain the stem cell compartment both in vivo and in intestinal organoids cultures [45–48]. Wnt/ $\beta$ -catenin signaling is also modulated by extracellular inhibitors that either bind and sequester Wnt proteins themselves (e.g., secreted Frizzled-related proteins or SFRP, Wnt-inhibitory factor, or WIF-1) or that physically interact with LRP receptors (e.g., Dickkopf or DKK), specifically inhibiting the canonical Wnt pathway [49].

As said before, most, if not all, sporadic CRC present an activated Wnt/ $\beta$ -catenin pathway, which is accepted as the initial step in colorectal tumorigenesis. Recurrent mutations include loss-of-function mutations in *APC* (>80%) and *RNF43* (10%), and gain-of-function mutations in *CTNNB1*/ $\beta$ -catenin (2%) and *RSPO2/3* (8%) [27,48,50,51]. Interestingly, alterations affecting these genes both in precancerous polyps and in tumors show marked mutual exclusivity [52]. Mutations in *APC* and *CTNNB1*/ $\beta$ -catenin lead to constitutive





**FIGURE 89.1** 1,25(OH)<sub>2</sub>D<sub>3</sub> exerts a multilevel antagonism of the Wnt/β-catenin signaling pathway in colon carcinoma cells. The pathway is activated upon binding of secreted Wnt factors to their plasma membrane Frizzled/LRP5/6 receptor complexes, which prevents the cytosolic degradation of β-catenin. Part of the accumulated β-catenin protein enters the cell nucleus where it acts as a transcriptional coactivator of TCF4-bound genes. 1,25(OH)<sub>2</sub>D<sub>3</sub> binding to VDR sequesters β-catenin, and therefore, it impedes the formation of transcriptionally active β-catenin/TCF4 complexes and thus, the induction of their target genes. Ligand-bound VDR upregulates the expression of *CDH1* encoding E-cadherin, which attracts cytosolic β-catenin to the plasma membrane *adherens junctions*, and of several genes including *DKK1*, *AXIN*, and *TCFL7* that contribute to inhibit the Wnt/β-catenin pathway. Additionally, 1,25(OH)<sub>2</sub>D<sub>3</sub> may inhibit the expression and secretion of IL-1β in neighbor macrophages, which activate Wnt signaling in carcinoma cells via the blockade of GSK-3β.

activation of the pathway, which is independent of Wnt ligands, while alterations in *RNF43* and *RSPO* amplify endogenous and otherwise intact Wnt/β-catenin signaling that is ligand-dependent [48]. The common consequence is the inhibition of β-catenin degradation and, therefore, the accumulation of free β-catenin. However, only a small number of CRC cells exhibit abundant β-catenin in the cytoplasm and nucleus. These cells are usually located at the tumor invasion front, in contact with the surrounding stroma. This apparent discrepancy with the model (known as the “β-catenin paradox”) suggests that in addition to mutations in components of the

destruction complex, β-catenin can be further activated by additional layers of regulation, which demonstrates the complexity of Wnt/β-catenin signaling deregulation in cancer ([53] and references therein). Accordingly, it has been demonstrated that effective nuclear accumulation of β-catenin requires additional mutations (e.g., *KRAS*) [54,55] or intensifying signals upstream the β-catenin destruction complex provided by Wnts, RSPOs, or hepatocyte growth factor (HGF) secreted by surrounding stromal cells [56,57]. In addition, loss of extracellular inhibitors of Wnt/β-catenin signaling, such as SFRP or DKK family members, is frequent in CRC and other

tumors and contributes to enhancing Wnt/ $\beta$ -catenin signaling by increasing nuclear  $\beta$ -catenin accumulation [58–62]. This results in activation of the  $\beta$ -catenin/TCF gene program that includes genes promoting proliferation and cell survival, such as *MYC* or *CCND1*/cyclin D1, as well as genes involved in loss of epithelial features and acquisition of migration capacity and other mesenchymal traits (e.g., *SNAI1/2*, *ZEB1*), a phenotypic change called EMT.

In the colon, Wnt factors and RSPOs are mostly secreted by pericryptal fibroblasts surrounding the bottom half of the colonic crypt and are essential to preserve the stem cell population that give rise to differentiation-committed transit amplifying (TA) cells. These cells move upward in the crypt where concentrations of Wnt ligands are lower, and when the Wnt/ $\beta$ -catenin pathway is eventually switched off, they undergo differentiation into absorptive enterocytes/colonocytes, mucosecretory goblet cells, or enteroendocrine cells [63–66]. Constitutive activation of the Wnt/ $\beta$ -catenin pathway due to mutation prevents TA cells differentiation and promotes the generation of adenomas/polyps [42,67]. Moreover, strong reactivation of the pathway in differentiated cells due to cooperation of inflammatory signals (e.g., NF- $\kappa$ B) with nuclear  $\beta$ -catenin results in dedifferentiation and entry into the cell cycle [68]. Likewise, loss of SMAD4 and activation of the Wnt/ $\beta$ -catenin pathway leads to dedifferentiation, cell cycle entry, reexpression of stem cell genes, and rapid adenoma formation in differentiated tissue [69].

However, despite the pivotal role that aberrant activation of the Wnt/ $\beta$ -catenin pathway plays in the development of CRC, targeting this pathway for clinical use remains elusive. Several special features may account for this including the lack of a feasible drug target (for example, kinases), the localization (nuclear) of  $\beta$ -catenin/TCF complexes, the plethora of activating factors (numerous Wnts, RSPOs, HGF, etc.), and the expected toxicity of potential inhibitors of this pathway due to the key role of Wnt/ $\beta$ -catenin signaling in many tissues and organs [36,43,70–73].

## 2. Human observational and intervention studies

A link between vitamin D and CRC is not surprising as the intestine is the organ with highest expression of the vitamin D receptor (VDR) and vitamin D is a key regulator of its physiology modulating many of its functions through the control of a high number of genes [74–76]. Some metaanalyses indicate that CRC is the neoplasia that is most clearly linked to vitamin D deficiency, but causality and the beneficial role of vitamin D in CRC prevention or treatment has not been fully demonstrated.

In 1980, Garland and Garland were the first to propose that the protective action of solar radiation on cancer risk is mediated by the vitamin D that it induces in skin [77]. Nine years later, a prospective study showed the inverse relation between the level of 25(OH)D in serum and CRC risk and mortality [78]. In recent decades, a large number of observational/epidemiological studies have analyzed the relation of solar irradiation, vitamin D dietary intake, and vitamin D status (circulating 25(OH)D) on total cancer and CRC incidence and mortality. In spite of their limitations (putative confounding effects of  $\text{Ca}^{2+}$  and other dietary components, enzymatic control of effective 1,25(OH) $_2$ D $_3$  concentrations in colon cells and the level of VDR expression, among others), many but not all studies indicate a protective action of vitamin D on CRC risk [1,79–83]. A metaanalysis supports that low plasma 25(OH)D level associates with poorer CRC patient survival, particularly in those with the VDR rs11568820 GG single-nucleotide polymorphism (SNP) [84]. Interestingly, a large study ( $n = 2592$ ) has revealed that the survival advantages in CRC patients with adequate vitamin D strongly depend on antioxidant status and are more pronounced in cases of low antioxidant capacity [85].

Trials on the effects of vitamin D supplementation on total cancer or CRC responsiveness and their results are controversial [86–89]. Unfortunately, published trials have major limitations or deficiencies: disparity to evaluate vitamin D status (sometimes by direct measures of circulating 25(OH)D and others indirectly by analyzing sun exposure, food intake composition, or vitamin D target genes expression), low doses of vitamin D that did not increase serum 25(OH)D concentration, calcium cotreatment, and insufficient analysis of vitamin D status and confounding factors [90–92]. Other limitations are low compliance, short duration, and follow-up, including only old population and questionable statistics analyses [93]. Some studies focused mainly on postmenopausal women, who probably do not represent the general population in terms of vitamin D responsiveness. Another common problem of these studies is a nonappropriated placebo group, with off-protocol vitamin D (and/or calcium) supplementation allowed. Furthermore, two major additional drawbacks common to most studies are that many participants were not deficient in 25(OH)D levels, and the lack of subgroup analysis. Clearly, (1) populations already replete of vitamin D are not expected to benefit from additional vitamin D treatment, and (2) the separate analysis of racial or body mass index subgroups may reveal beneficial action that are masked in global population analyses, for example, in the VITAL trial [93–98].

Moreover, few trials take into consideration individual genetics, polymorphisms in VDR, GC, CYP27B1,

*CYP24A1*, *DHCR7*, and *CYP2R1* genes that may modulate serum 25(OH)D levels and vitamin D action [86,99–101]. Thus, GC isoforms (rs4588 and rs2282679) may modulate the association of vitamin D status with mortality of CRC patients [100,102,103]. Globally, data on the importance of *VDR* genotype for CRC and other cancer types are conflicting, and it is not possible to rise any conclusion about the putative modulation by *VDR* polymorphisms of cancer risk, which may be modified by ethnicity and vitamin D status [104,105]. Remarkably, a comprehensive study using Mendelian randomization and a large set of variants (74 SNPs) suggests that low 25(OH)D level is unlikely to be a causal risk factor for most cancers [106].

An intriguing confounding factor is immune status, specifically the systemic inflammatory response. Vitamin D regulates many aspects of the immune function and has antiinflammatory effects; conversely, chronic inflammation, which is common in many types of cancer, is associated with low serum 25(OH)D concentrations [82,107]. These findings have led some authors to propose that many cancer patients have low serum 25(OH)D as a consequence of their neoplastic process or ad hoc treatment, that is, a reverse causality of the disease status [108]. Supporting this idea and the reduced response to vitamin D of severely ill patients at intensive care units, corticoids commonly given to these patients can inhibit the hepatic hydroxylation of vitamin D to 25(OH)D. Interestingly, Song and colleagues [109] reported that high plasma 25(OH)D level is associated with lower risk of the CRC subtype characterized by a high intratumoral periglandular immune/lymphocytic reaction. Further research is required to ascertain whether, as proposed by the authors, these results imply that host immunity may serve as a potential biomarker to predict benefits of vitamin D supplementation for CRC prevention.

Based on data from preclinical studies using cultured cells and animal models, several finished and ongoing clinical trials use combinations of vitamin D compounds with folic acid, omega-3 fatty acids, calcium, and antiinflammatory drugs (such as aspirin) or chemotherapeutic agents for CRC prevention or treatment. Furthermore, the long-term prevalent idea that vitamin D can be beneficial only in the early stages of cancer has been broadened and challenged by recent data on metastatic CRC patients: high circulating 25(OH)D levels in patients with CRC liver metastasis treated with percutaneous radiofrequency ablation predicted longer overall survival (OS) and time to recurrence [110] and improved also OS and progression-free survival (PFS) of metastatic CRC patients in a randomized phase III trial (CALGB/SWOG 80405) of chemotherapy plus bevacizumab (anti-VEGF antibody), cetuximab (anti-EGFR antibody), or both [90,111].

There is a clear need to wait for the results of well-designed randomized controlled trials selecting vitamin D-deficient patients, using appropriate placebo groups and vitamin D doses that adequately analyze baseline and follow-up vitamin D status, long follow-up, stratification of the results into subpopulations, and ideally individual vitamin D responsiveness. These studies are required to definitively assess the potential utility of *VDR* agonists, and to accept or reject the reverse causality of vitamin D deficiency in CRC and total cancer. Vitamin D-free placebos are, however, difficult to accomplish for ethical reasons, and, importantly, as discussed by Feldman and colleagues [112], there are still a number of questions that need to be addressed, including the optimal form of vitamin D (cholecalciferol, 1,25(OH)<sub>2</sub>D<sub>3</sub>, or analogs), doses, regimen (frequent low doses seem to be more efficient than large occasional bolus), and drug combination to be used.

Overall, available results suggest that vitamin D compounds, alone or more probably in combination with other agents, could help in the prevention and perhaps treatment of CRC. Observational and intervention studies and metaanalyses point toward an effect of vitamin D compounds reducing CRC progression and mortality, rather than on incidence [87,90–92,94,113–116]. Interestingly, a recent study has shown that high total vitamin D intake associates with decreased risks of EOCRC and precursors in a cohort of young women [1].

In support of a beneficial role of vitamin D in CRC, a large number of mechanistic preclinical studies, which are summarized in the following sections, show a variety of protective, antitumoral effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and other *VDR* agonists on CRC cells, several animal models of CRC, and, recently, also on CRC-associated fibroblasts and stem cells.

### 3. Expression of vitamin D hydroxylases and the vitamin D receptor in colorectal cancer

#### 3.1 Vitamin D hydroxylases

CRC cell responsiveness to vitamin D metabolites is mainly determined by the level of 1,25(OH)<sub>2</sub>D<sub>3</sub> within the cell and the degree of *VDR* expression. The intracellular level of 1,25(OH)<sub>2</sub>D<sub>3</sub> depends on the amount of circulating 25(OH)D and 1,25(OH)<sub>2</sub>D<sub>3</sub>, and on the expression and activity of the enzymes responsible for its synthesis (*CYP2R1*, *CYP27B1*) and degradation (*CYP24A1*).

Work by many groups show that normal and malignant colon epithelial cells contain different amounts of *CYP27B1* and *CYP24A1*. In 1997, Cross and colleagues showed that cultured CRC Caco-2 cells could synthesize



1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,24,25(OH)<sub>3</sub>D<sub>3</sub>, and 24,25(OH)<sub>2</sub>D<sub>3</sub> from 25(OH)D<sub>3</sub>, which suggests that they express both CYP27B1 and CYP24A1 enzymes [117]. Similar 25(OH)D<sub>3</sub> metabolism was found in primary cultures obtained from human CRC [118]. In addition, several studies reported that normal colon epithelial cells and most colorectal tumors express CYP27B1, and expression differences between normal and tumor tissues have been found [118–127]. Thus, it has been proposed that CYP27B1 expression is upregulated in moderately and well-differentiated human colorectal tumors and drastically decreases in poorly differentiated carcinomas [118,119,122,124,125]. Accordingly, CYP27B1 is expressed at similar levels in normal colonic epithelium as in aberrant crypt foci, polyps, and CRC irrespective of tumor differentiation, but its expression is lower in tumor cells metastasizing to regional lymph nodes [126]. More recently, CYP27B1 and CYP2R1 genes have been found upregulated in CRC samples compared with the adjacent nontumoral tissue [128]. In addition, SNPs in CYP27B1 such as rs28934604, rs58915677, rs13377933, and rs2229103 [129] or rs4646536 and rs10877013 [130,131] have been associated to the occurrence of CRC. In a later study, Latacz and colleagues have suggested a protective role of the T allele at the polymorphic site in rs10877012 (T/G) polymorphism in CYP27B1 gene in CRC patients [132]. In summary, available data suggest that CYP27B1 expression and 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis may be a protective mechanism of colon normal and cancerous cells to control their own growth autocrinally and/or paracrinally. This mechanism is lost in advanced CRC [119,124,125,133].

In contrast to CYP27B1, a number of studies have reported that CYP24A1 is expressed at low levels in normal colon epithelial cells, whereas it is overexpressed in colorectal tumors [118,125,134–137]. Remarkably, CYP24A1 upregulation seems to be an early event in CRC progression, as it has already been observed in a proportion of colorectal adenomas [135]. Moreover, CYP24A1 expression in colorectal tumors strongly correlates with that of the proliferation markers Ki67, CDC6, MCM2, MCM4, and MCM7, which suggests that elevated CYP24A1 levels may provide a growth advantage to the tumor [135,136]. In line with this, cultured and xenografted CRC cells overexpressing CYP24A1 proliferate faster than controls, and the tumors that they generate in immunosuppressed mice are more aggressive [138]. Regarding the mechanism underlying CYP24A1 upregulation, Höbaus and colleagues have reported that 60% of CRC show an increase in the CYP24A1 gene copy number and that this number directly correlates with CYP24A1 expression [136]. Similarly, a study using quantitative measurement of the DNA copy number across amplified regions had previously proposed CYP24A1 as a candidate oncogene in breast cancer [139]. Höbaus and

colleagues did not find differences in the methylation status of the CYP24A1 gene promoter between normal and tumor colon tissue or between colorectal tumors with high or low CYP24A1 expression, thus excluding DNA hypomethylation as a possible cause of CYP24A1 upregulation in CRC [136].

Polymorphisms in CYP24A1 gene affect the response to vitamin D supplementation in CRC patients [140]. A study analyzing the copy number variation in Chinese population indicated that in 51.1% of CRC samples, CYP24A1 was amplified when compared with matched adjacent normal tissues [141]. In an African American US cohort, rs6022990 SNP in the CYP24A1 gene was linked with left-sided CRC ( $P = 0.018$ ) [142], while four other SNPs showed a correlation with the risk of CRC [143] and the rs4809957 A > G polymorphism may lead to a worse prognosis [130]. Recently, Chain and colleagues have reported that some CYP24A1 polymorphisms in CRC patients of the Jiamusi population were significantly associated with clinical features such as poor differentiation or tumor type [144]. Another study has identified squalene epoxidase, an enzyme that controls cholesterol biosynthesis and is upregulated in CRC associated with poor prognosis, as a strong inducer of CYP24A1 and MAPK signaling [145].

High CYP24A1 expression in CRC cells must lead to the depletion of intracellular 1,25(OH)<sub>2</sub>D<sub>3</sub> levels through their rapid conversion into less active metabolites, thus limiting the antitumor effects of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Interestingly, a combination treatment using 1,25(OH)<sub>2</sub>D<sub>3</sub> and CYP24A1 inhibitors exerts a significantly greater inhibition of CRC cell proliferation than 1,25(OH)<sub>2</sub>D<sub>3</sub> alone. This suggests that such combination therapy should be explored for CYP24A1-overexpressing colorectal tumors to extend the bioavailability of 1,25(OH)<sub>2</sub>D<sub>3</sub> [138,146]. In general, the downregulation of CYP27B1 and the upregulation of CYP24A1 found in advanced CRC would lead to attenuated 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and accelerated 1,25(OH)<sub>2</sub>D<sub>3</sub> catabolism, thus reducing VDR activation and causing partial or total resistance to the antitumor effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> [118,133].

### 3.2 Vitamin D receptor

Cellular response to 1,25(OH)<sub>2</sub>D<sub>3</sub> crucially depends on VDR expression levels. Normal colon epithelial cells and several CRC cell lines express VDR and respond to 1,25(OH)<sub>2</sub>D<sub>3</sub>. However, certain CRC cell lines have lost VDR expression and thus have become 1,25(OH)<sub>2</sub>D<sub>3</sub>-resistant [147–150]. Remarkably, well-differentiated CRC cell lines display higher VDR levels than poorly differentiated cells [147]. Several studies have analyzed VDR expression in human CRC biopsies and compared it with that found in normal colonic tissue. Overall, the results suggest that VDR expression is induced in



precancerous lesions and early steps of colorectal tumorigenesis (aberrant crypt foci, polyps/adenomas), but decreases in advanced stages (carcinomas) [119,126,127,134,137,147,151–158]. Moreover, VDR levels are extremely low in CRC lymph node metastases [126]. Accordingly, elevated VDR expression in primary tumors is associated with high differentiation, absence of node involvement, and good prognosis in CRC [153,156,158–160]. VDR levels are also reduced in the colonic mucosa of UC patients. Remarkably, VDR expression is significantly decreased in long-term patients (over 10 years) and in patients with UC-associated dysplasia or CRC [149].

As a cellular response to  $1,25(\text{OH})_2\text{D}_3$  requires VDR expression, the downregulation of VDR observed in advanced CRC must generate tumor resistance to a therapy with vitamin D compounds and to endogenously synthesized  $1,25(\text{OH})_2\text{D}_3$ . It is, however, worth noting that studies mentioned referred only to carcinoma cells and not to stromal cells. The analysis of the expression of VDR in 658 patients with CRC with prolonged clinical follow-up indicated that high VDR expression in stromal fibroblasts is associated with OS and PFS independently of the expression in carcinoma cells, with important effects at the level of gene expression that will be discussed in the following sections [160]. Conversely, CRC patients have higher adipose tissue VDR mRNA and protein levels than healthy individuals, which may indicate a compensatory mechanism in response to the low blood  $25(\text{OH})\text{D}$  levels in these patients [161].

Our group has studied the mechanisms responsible for VDR downregulation during CRC progression. We reported that the transcription factor SNAIL1, encoded by the *SNAIL1* gene, binds to three E-boxes in the proximal human *VDR* gene promoter and represses its expression in CRC cells. In addition, SNAIL1 reduces *VDR* RNA half-life [155]. Subsequently, we found that, in addition to SNAIL1, its family member SNAIL2 (also known as SLUG, encoded by the *SNAIL2* gene) represses VDR expression through the same E-boxes in the *VDR* gene promoter, and blocks the antitumor effects of  $1,25(\text{OH})_2\text{D}_3$  in CRC cells. Moreover, SNAIL1 and SNAIL2 show an additive repressive effect on the activity of the *VDR* gene promoter [158]. In line with this, SNAIL2/SLUG inhibits the enhanced sensitivity to radiation mediated by  $1,25(\text{OH})_2\text{D}_3$  in DLD-1 and HCT116 CRC cells [162].

SNAIL1 and SNAIL2 are inducers of EMT, a process that takes place physiologically in several developmental situations such as mesoderm formation and neural crest migration and is reactivated in adults in certain conditions such as wound healing, fibrosis, and cancer. Through this process, epithelial cells that overexpress these transcription factors undergo gene expression

reprogramming characterized by the downregulation of epithelial genes and the induction of mesenchymal genes. This leads to a drastic phenotypic change from highly compact, polarized, and adhesive epithelial cells to motile individual mesenchymal cells that can degrade the extracellular matrix and develop a migratory and invasive phenotype [163–167]. We found that nearly 75% of human colorectal tumors exhibit higher SNAIL1 and/or SNAIL2 levels than those observed in normal colonic tissue and that this upregulation is significantly associated with diminished VDR expression. Indeed, the lowest VDR levels were detected in those tumors that overexpress both transcription factors [155,156,158]. We also showed that elevated SNAIL1 expression in colon tumors is associated with VDR downregulation in the histologically normal tissue adjacent to the tumor, which suggests that SNAIL1-expressing colorectal tumors secrete signals that paracrinally modulate VDR expression in neighboring cells [168]. Other studies further support the repression of VDR by SNAIL factors in CRC. Silibinin, a flavonolignan with anticancer properties that downregulates SNAIL1 and SNAIL2, increases VDR levels and restores  $1,25(\text{OH})_2\text{D}_3$  antitumor action in  $1,25(\text{OH})_2\text{D}_3$ -resistant CRC cells [169]. Similarly, the reduced SNAIL1 expression detected in colon tumors that have been chemically induced in mice carrying an *Egfr* hypomorphic mutation (*Egfr*<sup>wa2/wa2</sup>) is accompanied by *Vdr* upregulation [170]. In addition, Knackstedt and colleagues described that the low colonic *Vdr* expression observed in a murine model for acute colitis is associated with an increase in *Snail* [171].

These data indicate that the induction of SNAIL1 and/or SNAIL2 during CRC progression is responsible for VDR downregulation. The expression of both transcription factors is probably required to achieve maximum VDR repression. Our results also imply that CRC patients with high tumor expression of SNAIL1 and/or SNAIL2 would be poor responders to the antitumor effects of  $1,25(\text{OH})_2\text{D}_3$  compounds. Since these factors are frequently upregulated in advanced CRC (associated with the acquisition of migratory and invasive properties by the tumor cells), it was previously thought that VDR agonists should be preferentially used in CRC as preventive agents in high-risk populations and/or as therapeutic drugs for patients in early stages of the disease. Interestingly, we have reported that CRC-associated fibroblasts express VDR and that  $1,25(\text{OH})_2\text{D}_3$  treatment inhibits their protumoral properties and phenotype. Accordingly, high VDR expression in these fibroblasts is significantly associated with a favorable clinical outcome in a large cohort of metastatic CRC patients [160]. These results substantially broaden the vision of the applicability of VDR agonists for CRC, as they suggest that these compounds could exert

therapeutic anticancer effects in CRC patients whose tumor fibroblasts express VDR even if their carcinoma cells lack VDR expression.

The repression of VDR by transcription factors of the SNAIL family is not exclusive to CRC. SNAIL1 and SNAIL2 have been described to downregulate VDR expression and block the antitumor action of 1,25(OH)<sub>2</sub>D<sub>3</sub> in human osteosarcoma and breast cancer cells, respectively [172,173]. As SNAIL factors are overexpressed in several neoplasias [174,175] and reduced VDR levels have been found in a variable proportion of melanomas and breast, lung, and ovarian tumors [134,176–179], it is reasonable to propose that SNAIL proteins may also be involved in VDR downregulation in these malignancies.

We found that other transcription factors that are also inducers of EMT, such as ZEB1, ZEB2, TWIST1, or E47, do not modify the activity of the proximal human VDR gene promoter in SW480-ADH CRC cells [158]. However, Lazarova and colleagues reported that ZEB1 binds to two distal E-boxes in the murine *Vdr* promoter and induces its expression in human SW620 CRC cells, but not in other CRC cells such as HCT116 [180]. Moreover, studies using different CRC patient cohorts show an absence of correlation or a significant direct correlation between ZEB1 and VDR expression in colon tumors [156,168,181]. Furthermore, Peña and colleagues described a stronger direct correlation in colorectal tumors with elevated expression of the transcriptional coactivator p300 [181]. In addition, Maurer and colleagues reported that the transcription factor WT1 potentiates 1,25(OH)<sub>2</sub>D<sub>3</sub> action by inducing VDR transcription in HT29 CRC cells [182].

Several evidence suggest that VDR posttranscriptional repression by microRNAs (miRs) may also be involved in the reduction of VDR levels found in CRC. *miR-27b* and *miR-298* inhibit VDR expression in LS180 CRC cells [183], and *miR-346* mediates the downregulation of VDR expression promoted by the proinflammatory cytokine TNF- $\alpha$  in HCT116 CRC cells [184]. Accordingly, VDR repression correlates with high TNF- $\alpha$  and *miR-346* levels in an experimental colitis mouse model and in human biopsies from patients with IBDs [184]. In addition, *miR-125b*, which is overexpressed in CRC metastases [185], downregulates VDR expression and reduces 1,25(OH)<sub>2</sub>D<sub>3</sub> action in breast cancer cells [186]. The expression of the long noncoding (lnc)RNA *H19* negatively correlates with that of VDR in primary colon tissues and CRC cells, and its overexpression reduces VDR expression through *miR675-5p* [187], while in Caco-2 cells, *miR-372/miR-373* repress VDR expression [188].

VDR expression is also subjected to regulation by RNA-binding proteins. HuR binds to mouse *Vdr* RNA via its 3'-untranslated region (UTR) and enhances its translation in intestinal epithelial cells [73]. Conversely,

the zinc finger protein 36 (ZFP36) has been reported to bind the 3'-UTR of VDR mRNA, leading to its degradation in colonic epithelial cells under inflammatory conditions [189].

Interestingly, Kure and colleagues showed that *KRAS* and *PIK3CA* activating mutations, which are, respectively, found in 30%–45% and 10%–20% of human CRC, are independently associated with high tumor VDR expression [157]. In contrast, two other studies reported that transforming *Hras* inhibits Vdr expression in murine HC11 mammary cells and NIH3T3 fibroblasts [190–192]. This implies species-, cell type-, and/or family member-dependent regulation of VDR by RAS. Nearly half of human CRC harbor *TP53* inactivating mutations. While Kure and colleagues did not find a statistical association between the expression of p53 and VDR proteins in a cohort of CRC patients [157], Maruyama and colleagues showed that p53 and its family members p63 and p73 induce VDR expression in cultured CRC cells [193]. Notably, mutated p53 interacts with VDR, increases nuclear VDR levels, and modulates the 1,25(OH)<sub>2</sub>D<sub>3</sub> response in colon and other types of cancer cells, changing 1,25(OH)<sub>2</sub>D<sub>3</sub> from proapoptotic to antiapoptotic [194]. Moreover, a positive correlation between VDR and CDX2 has been reported, and low expression of VDR or CDX2 is associated with the sensitivity to chemotherapy and BRAF and PI3K-mTOR inhibitors [195]. Together, these data suggest that the mutational status of CRC patients may influence their response to therapies based on VDR agonists.

Several SNPs of VDR gene have been studied in relation to CRC occurrence and prognosis, but only few of them seem to affect the expression of the VDR gene in relation of CRC. There was not a correlation between *BsmI*, *FokI*, *ApaI*, and *TaqI* VDR polymorphisms and CRC risk in the overall analyses. However, *ApaI* and *BsmI* loci are associated with CRC risk in elderly and female patients, respectively, in Saudi population [196]. Another study indicated that *ApaI* and *TaqI* VDR SNPs increase, while *BsmI* reduces the risk of CRC in Saudi population [197]. Similarly, in the Newfoundland population, a multiple testing adjustment revealed a trend for an association between VDR *BsmI* polymorphism rs1544410 and OS of CRC patients (*p*-adjusted = 0.058): G-allele was related to worse OS in comparison with the A-allele [198]. Fedirko and colleagues did not find an association between VDR (*FokI* and *BsmI*) and survival [199]. Conversely, Cho and colleagues studied the association of VDR *FokI* polymorphism with CRC risk and found an inverse association of f allele [200]. Messaritakis and colleagues found a significant association between all these four polymorphisms of VDR and CRC, significantly associated with stage IV, *KRAS* mutations, and Toll-like receptor (TLR) genetic variants. Besides, using multivariate analysis, they found that tt,

aa, and ff genotypes are independent factors associated with decreased OS [201]. Although no SNPs individually affect the risk of CRC, taB and tAb might increase the possibility of CRC development [202].

Some natural compounds have been reported to modulate the colonic vitamin D system. The steroid 17 $\beta$ -estradiol and certain phytoestrogens increase VDR and CYP27B1 expression but reduce that of CYP24A1 in cultured CRC cells and in the mouse and rat colon [203–208]. Accordingly, 17 $\beta$ -estradiol-based postmenopausal hormone replacement therapy induces VDR expression in the human rectal mucosa [209]. Dietary calcium may also regulate the colonic vitamin D system, as a low-calcium diet significantly increases CYP24A1 expression in the mouse colon [125,210–212], while reduced calcium concentration in the culture medium of Caco-2/AQ CRC cells induces VDR levels [211]. Moreover, a randomized, double-blinded placebo-controlled clinical trial indicated that calcium supplementation for 6 months reduced the expression of CYP24A1 in the normal-appearing rectal mucosa of colorectal adenoma patients [213]. Remarkably, a soy-enriched diet (which contains high levels of phytoestrogens) counteracts the increase of colonic CYP24A1 expression promoted by low dietary calcium in mice [214]. Furthermore, the short-chain fatty acid butyrate increases VDR expression and enhances the prodifferentiation action of 1,25(OH) $_2$ D $_3$  in human CRC cells [215,216]. Therefore, CRC patients' nutritional status and the use of these compounds in combination with 1,25(OH) $_2$ D $_3$  analogs may modulate tumor responsiveness to 1,25(OH) $_2$ D $_3$ , and potentially reduce the putative resistance caused by the alteration of the vitamin D system at advanced stages of CRC progression.

## 4. Mechanism of action of 1,25(OH) $_2$ D $_3$ in colorectal cancer

### 4.1 Antagonism of the Wnt/ $\beta$ -catenin pathway by 1,25(OH) $_2$ D $_3$ in colon cancer

Our group was a pioneer in the discovery of antagonism between 1,25(OH) $_2$ D $_3$ /VDR and the Wnt/ $\beta$ -catenin signaling pathway in CRC cells [148]. The key role of this pathway for CRC initiation and progression (see Sections 1.2 and 1.3) makes this finding highly relevant. In recent years, we and others have revealed a series of mechanisms by which 1,25(OH) $_2$ D $_3$  interferes the Wnt/ $\beta$ -catenin signaling (Fig. 89.1).

#### 4.1.1 Enhancement of VDR- $\beta$ -catenin protein–protein interaction

VDR physically interacts with  $\beta$ -catenin inside the cell nucleus, and in this way, it competes and interferes

the binding between  $\beta$ -catenin and TCF [148]. This results in a decrease in the number of transcriptionally active  $\beta$ -catenin/TCF complexes, which hampers the expression of  $\beta$ -catenin/TCF target genes such as *MYC*, *TCF1*, *PPARG*, *CD44*, *LEF1*, and *AXIN2*. Interestingly, although VDR- $\beta$ -catenin interaction is exacerbated by 1,25(OH) $_2$ D $_3$ , it also takes place in the absence of the hormone, which suggests that VDR binding to  $\beta$ -catenin is partially independent of 1,25(OH) $_2$ D $_3$  [148].

These findings were later confirmed by Shah and colleagues, who showed that the C-terminal region of  $\beta$ -catenin and the C-terminal activator function-2 (AF-2) motif of VDR were the main domains involved in the interaction [217]. In addition, Egan and colleagues reported that binding between  $\beta$ -catenin and VDR was potentiated by the wild-type APC protein and that the VDR ligand lithocholic acid (LCA) also enhanced this interaction, although less strongly than 1,25(OH) $_2$ D $_3$  [218]. Nevertheless, despite the antagonistic effect of 1,25(OH) $_2$ D $_3$ /VDR on Wnt/ $\beta$ -catenin signaling,  $\beta$ -catenin potentiates—rather than hampers—VDR-mediated transcription [148,217,218].

#### 4.1.2 Induction of E-cadherin

Loss of E-cadherin expression is frequent during the early progression of different types of cancers and is associated with the acquisition of invasive properties by tumor cells. Therefore, E-cadherin is considered a suppressor of invasion [219]. We reported that 1,25(OH) $_2$ D $_3$  upregulates *CDH1*/E-cadherin in CRC cells by a transcriptional mechanism that requires protein synthesis de novo [148]. In addition, we found a direct correlation between VDR and *CDH1*/E-cadherin RNA levels in human colon tumors [148,156]. Concomitant with the acquisition of a more differentiated phenotype, 1,25(OH) $_2$ D $_3$ -mediated expression of E-cadherin also resulted in redistribution of  $\beta$ -catenin from the nucleus of CRC cells to the plasma membrane *adherens junctions* where it binds to the cytoplasmic tail of E-cadherin. Consequently, in the presence of 1,25(OH) $_2$ D $_3$ , fewer molecules of  $\beta$ -catenin were available in the nucleus for TCF binding, which led to reduced expression of  $\beta$ -catenin/TCF target genes [148]. Thus, *CDH1*/E-cadherin induction has prodifferentiation, antiproliferative, and antimigratory effects that probably contribute to the antitumoral action of 1,25(OH) $_2$ D $_3$  in CRC. Remarkably, similar results have been reported by Gröschel and colleagues using the nonmalignant colonic adenoma cell line LT97 [220].

Several mechanisms contribute to the upregulation of *CDH1*/E-cadherin by 1,25(OH) $_2$ D $_3$  in CRC cells including the activation of the RhoA-ROCK and p38MAPK-MSK1 protein kinase pathway [221], the expression of the protease inhibitor cystatin D [222], the inhibition of the Sprouty receptor tyrosine kinase



signaling antagonist-2 (*SPRY2*) gene [223], and the induction of the KDM6B/Jumonji C (JmJC) domain-containing protein (JMJD) 3 histone H3 lysine demethylase [224]. Interestingly, activation of most of these effectors was also required for Wnt/ $\beta$ -catenin antagonism by 1,25(OH)<sub>2</sub>D<sub>3</sub>. This supports a close relation between E-cadherin upregulation and inhibition of  $\beta$ -catenin/TCF-mediated transcription. However, induction of E-cadherin is not an absolute requirement for 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated antagonism of Wnt/ $\beta$ -catenin signaling, since this has also been observed in the E-cadherin-negative LS174T cell line [148]. Induction of E-cadherin by 1,25(OH)<sub>2</sub>D<sub>3</sub> and concomitant inhibition of Wnt/ $\beta$ -catenin signaling have also been reported in other cell types [225,226].

As a result of its repressive effect on VDR expression, SNAIL1 blocks the induction of E-cadherin expression and the acquisition of an epithelial phenotype promoted by 1,25(OH)<sub>2</sub>D<sub>3</sub>. Consequently, in SNAIL1-expressing cells treated with 1,25(OH)<sub>2</sub>D<sub>3</sub>,  $\beta$ -catenin does not translocate from the nucleus to the *adherens junctions* at the plasma membrane, and Wnt/ $\beta$ -catenin signaling remains active. Accordingly, SNAIL1 abrogates the inhibitory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the proliferation and migration of cultured CRC cells and the antitumor action of the 1,25(OH)<sub>2</sub>D<sub>3</sub> analog EB1089 in such cells xenografted in immunosuppressed mice [155,227].

#### 4.1.3 Regulation of DKK1 and DKK4

1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of the *DKK1* gene in CRC cells, and there is a significant direct correlation between VDR and *DKK1* RNA expression in tumor biopsies from CRC patients [228]. In line with this, dietary vitamin D intake is inversely associated with *DKK1* promoter methylation in a large cohort of CRC patients [229]. As said before, *DKK1* is a member of the *DICKKOPF* gene family that encodes secreted proteins that bind to LRP5/6 and function as extracellular inhibitors of Wnt/ $\beta$ -catenin signaling [230]. Binding of *DKK1* to LRP5/6 blocks the interaction between Wnt, Frizzled, and LRP5/6 and instead induces the formation of a complex with another *DKK1* receptor named Kremen, which leads to LRP5/6 endocytosis [231,232]. Both mechanisms result in the inhibition of Wnt/ $\beta$ -catenin signaling.

Upregulation of *DKK1* by 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells is expected to have greatest impact in Wnt ligand-dependent tumors such as those with mutations in *RNF43* or *RSPO2/3*, while it should be less relevant in tumors with a constitutive active Wnt/ $\beta$ -catenin pathway due to mutations in intracellular effectors such as *APC* or *CTNNB1*/ $\beta$ -catenin. However, it has been shown that CRC cells can respond to exogenous and autocrine Wnt ligands that further activate the Wnt/ $\beta$ -catenin signaling pathway despite having mutations that

stabilize  $\beta$ -catenin [57]. Moreover, our group and others have suggested that *DKK1* might have antitumor effects that are independent of Wnt/ $\beta$ -catenin inhibition [58,233–235], and we have also demonstrated that a proportion of *DKK1* protein is present within the nuclei of CRC cells and healthy small intestine and colon mucosa cells. Nuclear *DKK1* protein locates at specific chromatin sites of active transcription and regulates several cancer-related genes that are involved in the detoxification of chemotherapeutic agents including the cancer stem cell marker aldehyde dehydrogenase 1A1 (*ALDH1A1*) and Ral-binding protein 1-associated Eps domain-containing 2 (*REPS2*) [236]. *DKK1* expression is lost during CRC progression [61,236], partly due to gene promoter methylation [58,60,237,238], which suggests a tumor suppressor role. However, we have demonstrated that nuclear *DKK1* expression remains high in around 15% of CRC patients and is associated with decreased PFS after chemotherapy administration and OS [236]. Therefore, we proposed that nuclear *DKK1* is a marker of chemoresistance at later stages of CRC progression, when VDR expression is lost in a high proportion of tumors.

Interestingly, Postigo and colleagues have reported that EMT transcription factor ZEB1 cooperates with  $\beta$ -catenin/TCF in inducing *DKK1* expression, which leads to downregulation of senescence-associated histone variant macroH2A1 promoting senescence escape and EMT. This ZEB1/*DKK1* pathway engages also mutant p53, MDM2, and CtBP [239]. These authors have also shown that high expression of ZEB1 and *DKK1* predicted poor outcome in a group of patients in which macroH2A1 expression was low [239,240].

*DKK4*, another *DICKKOPF* family member, is repressed by 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells by a transcriptional mechanism involving binding of VDR and SMRT corepressor to a region adjacent to its transcription start site [241]. Notably, our group and others have shown that despite its reported function as a weak Wnt inhibitor, *DKK4* is overexpressed in human colon tumors and in IBD [241–244] and that an inverse correlation exists between the levels of VDR and *DKK4* RNA in human colorectal tumors [241]. In line with this, ectopic expression of *DKK4* increased the migration, invasion, and proangiogenic properties of CRC cells [241], and it has been found to enhance resistance to chemotherapeutics in CRC cells [245], which suggests that the inhibition of *DKK4* by 1,25(OH)<sub>2</sub>D<sub>3</sub> may contribute to the antitumor effects of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

In summary, available data indicate that *DKK* proteins are a family of Wnt antagonists with additional functions whose pharmacological potential as cancer therapeutics remains unclear but that seems to mediate some of the protective actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC [246].



The mechanisms of Wnt/ $\beta$ -catenin antagonism reported before are completely dependent on VDR expression, as they do not take place in VDR-negative human CRC cells (SW480-R and SW620) or in VDR-positive cells (SW480-ADH) in which expression of VDR has been silenced either by using specific shRNA or by ectopic expression of SNAIL1 or SNAIL2 [148,155,158,227,228,247]. Since SNAIL1 is induced by Wnt/ $\beta$ -catenin signaling [248–250], it may constitute a mechanism to bypass the inhibitory effect of  $1,25(\text{OH})_2\text{D}_3$  on this pathway. In addition, we have found that several less calcemic analogs of  $1,25(\text{OH})_2\text{D}_3$ , such as EB1089, KH1060, MC903, WU515, CD578, and WY1113, antagonize Wnt/ $\beta$ -catenin signaling in human CRC cells to a similar or even greater extent than the hormone itself [148,251]. Likewise, Xu and colleagues reported similar results with the BTW and QW analogs in breast and CRC cells [225].

#### 4.1.4 Other mechanisms of Wnt/ $\beta$ -catenin antagonism by $1,25(\text{OH})_2\text{D}_3$ in cultured carcinoma cells

Beildeck and colleagues have reported that  $1,25(\text{OH})_2\text{D}_3$  induces the expression of the *TCF7L2/TCF4* gene in CRC cells by a VDR-dependent indirect mechanism and also that Tcf4 protein levels were lower in the mammary gland of *Vdr*<sup>-/-</sup> mice than in wild-type mice [252]. These authors suggested that upregulation of TCF4 might have a protective role on CRC since it has been reported that TCF4 inhibits the growth of CRC cells [253]. In contrast, Gröschel and colleagues showed that the colon of healthy mice that were fed a high vitamin D diet showed reduced expression of both  $\beta$ -catenin and TCF4 [220]. They also found that  $1,25(\text{OH})_2\text{D}_3$  diminishes the nuclear levels of  $\beta$ -catenin in LT97 colon microadenoma cells, and thus, it inhibits the expression of Wnt target genes such as *BCL2*, *CCND1/cyclin D1*, *SNAIL1*, *CD44*, and *LGR5* [220]. In this regard, Li and colleagues have recently reported that  $1,25(\text{OH})_2\text{D}_3$  suppressed gastric cancer cell growth through downregulating *CD44* expression, which is concomitant to the inhibition of the Wnt/ $\beta$ -catenin pathway [254].

Calcipotriol, a potent nonhypercalcemic  $1,25(\text{OH})_2\text{D}_3$  analog, inhibits Wnt/ $\beta$ -catenin signaling and decreases the level of the Wnt coreceptor LRP6 in pancreatic ductal adenocarcinoma cell lines, through a mechanism involving transcriptional upregulation of the low-density lipoprotein receptor adaptor protein 1 [255]. By contrast,  $1,25(\text{OH})_2\text{D}_3$ /VDR has been shown to induce the Wnt inhibitor *AXIN1* in CRC cells through a VDRE located in the gene promoter [256]. Interestingly, it has been reported that VDR upregulates the expression of long noncoding RNA (lncRNA) *TOPORS antisense RNA 1 (TOPORS-AS1)* in ovarian cancer, which in turn inhibits Wnt/ $\beta$ -catenin signaling by increasing

$\beta$ -catenin phosphorylation and suppressing the expression of *CTNNB1/ $\beta$ -catenin*. This mechanism requires the RNA-binding protein heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1). Overexpression of *TOPORS-AS1* in ovarian cancer cells suppressed cell proliferation and inhibited migration, invasion, and colony formation, and patients with high *TOPORS-AS1* expression had better overall survival compared with low expression ones [257].

Kaler and colleagues assigned a role to the tumor stroma in the interplay between Wnt/ $\beta$ -catenin signaling and  $1,25(\text{OH})_2\text{D}_3$  [258]. They reported that interleukin (IL)-1 $\beta$  secreted by THP-1 macrophages in response to signals coming from the tumor inhibited GSK-3 $\beta$  activity in CRC cells. This resulted in  $\beta$ -catenin stabilization and increased  $\beta$ -catenin/TCF transcriptional activity. Moreover, activation of Wnt/ $\beta$ -catenin signaling in these cells led to stabilization of SNAIL1 and protected them from TRAIL-mediated apoptosis [259]. These authors found that  $1,25(\text{OH})_2\text{D}_3$  repressed the expression of IL-1 $\beta$  in THP-1 macrophages, thus blocking activation of the Wnt/ $\beta$ -catenin pathway in tumor cells and sensitizing them to TRAIL-mediated apoptosis [258,259].

Notably, Meyer and colleagues analyzed the overlap between  $\beta$ -catenin/TCF and VDR/RXR cistromes in CRC cells and found that the two heterodimers colocalize at 74 sites that lie near a limited set of genes including those of *FOS* and *MYC* proto-oncogenes. These data suggest possible transcriptional antagonism of both complexes at certain gene loci [260]. In fact, *MYC* plays a pivotal role in the cross-talk between  $1,25(\text{OH})_2\text{D}_3$  and the Wnt/ $\beta$ -catenin pathway. On the one hand, ligand-activated VDR inhibits *MYC* expression by direct interaction with two VDREs in the promoter region [260,261]. On the other hand, the antagonism of  $1,25(\text{OH})_2\text{D}_3$  on Wnt/ $\beta$ -catenin signaling prevents the transcription of *MYC* mediated by  $\beta$ -catenin/TCF through several Wnt-responsive elements (WRE) at the *MYC* promoter [148,262].

#### 4.1.5 Wnt/ $\beta$ -catenin antagonism by $1,25(\text{OH})_2\text{D}_3$ in animal models and clinical trials

Several studies using animal models to establish the protective action of  $1,25(\text{OH})_2\text{D}_3$  and nonhypercalcemic analogs on CRC have assessed the status of the Wnt/ $\beta$ -catenin pathway. Most, if not all, of these studies confirmed an antagonistic action on this pathway. A clinical trial conducted by Ahearn and colleagues was designed to study the effect of 6 months of daily supplementation with vitamin D<sub>3</sub> (800 IU) and/or calcium (2 g) on the expression of the Wnt/ $\beta$ -catenin pathway proteins APC,  $\beta$ -catenin, and E-cadherin in crypts of normal rectal mucosa from patients with sporadic colorectal adenoma [263]. Both vitamin D<sub>3</sub> and calcium individually

increased the expression of APC and reduced that of  $\beta$ -catenin in the differentiated upper half of the crypts, which resulted in a higher APC/ $\beta$ -catenin ratio. Moreover, supplementation with vitamin D<sub>3</sub> also led to an increase in E-cadherin [263]. These results were mostly confirmed in a subsequent 1-year follow-up clinical trial using daily supplementation with vitamin D<sub>3</sub> (1000 IU) and/or calcium (1.2 g) [264]. In line with this, Juniku-Shkololli and colleagues showed that the average vitamin D levels in CRC patients with positive nuclear  $\beta$ -catenin were lower than those of patients with negative nuclear  $\beta$ -catenin [265]. Altogether, these results confirm those obtained in culture cells and animal models and support the hypothesis that 1,25(OH)<sub>2</sub>D<sub>3</sub> affects Wnt/ $\beta$ -catenin signaling in a way that is protective in CRC. Interestingly, a study has shown that in 67 CRC patients, high circulating 25(OH)D<sub>3</sub> levels associate with low promoter methylation of the extracellular Wnt inhibitor *SFRP2* gene, suggesting that vitamin D might also antagonize the Wnt/ $\beta$ -catenin pathway through epigenetic mechanisms [266]. In another study, the RNA expression levels of VDR and Wnt-inhibitory factor (WIF)1 correlated directly and were found low in CRC tissues in comparison with their marginal tissues [267]. Curiously, stabilization of  $\beta$ -catenin protein expands mast cells to promote high incidence of colon polypoidosis in mice [268]. This suggests an indirect contribution of  $\beta$ -catenin signaling in mast cells to their maturation and to increased risk of CRC.

#### 4.1.6 Antagonism of 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR by the Wnt/ $\beta$ -catenin pathway

In addition to 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibition of the Wnt/ $\beta$ -catenin pathway, there is also evidence of a bidirectional cross-talk, and thus, that Wnt/ $\beta$ -catenin signaling may also interfere the actions of 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR. A mechanism by which Wnt/ $\beta$ -catenin prevents 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR-mediated effects is the induction of the EMT transcription factors SNAIL1 and SNAIL2. Both are phosphorylation targets of GSK-3 $\beta$ , which tags them for  $\beta$ -TrCP-mediated ubiquitination and subsequent proteasomal degradation [248,269,270]. Therefore, Wnt-induced inhibition of GSK-3 $\beta$  results in stabilization of SNAIL proteins. On top of that, inactivation of GSK-3 $\beta$  also induces *SNAIL1* transcription through a mechanism that involves NF- $\kappa$ B [271]. Moreover, the product of the  $\beta$ -catenin/TCF target gene *AXIN2* promotes SNAIL1 stabilization at least in breast cancer cells by controlling GSK-3 $\beta$  localization. When levels of Axin2 increase, GSK-3 $\beta$  is excluded from the nucleus and Snail1 remains in a nonphosphorylated, transcriptionally active state [272]. Additionally, Wu and colleagues have reported that Wnt3A upregulates *SNAIL2* gene expression in breast cancer cells [273]. SNAIL proteins are important negative regulators of

VDR expression, and their regulation by Wnt/ $\beta$ -catenin signaling may be instrumental for the control of 1,25(OH)<sub>2</sub>D<sub>3</sub> actions.

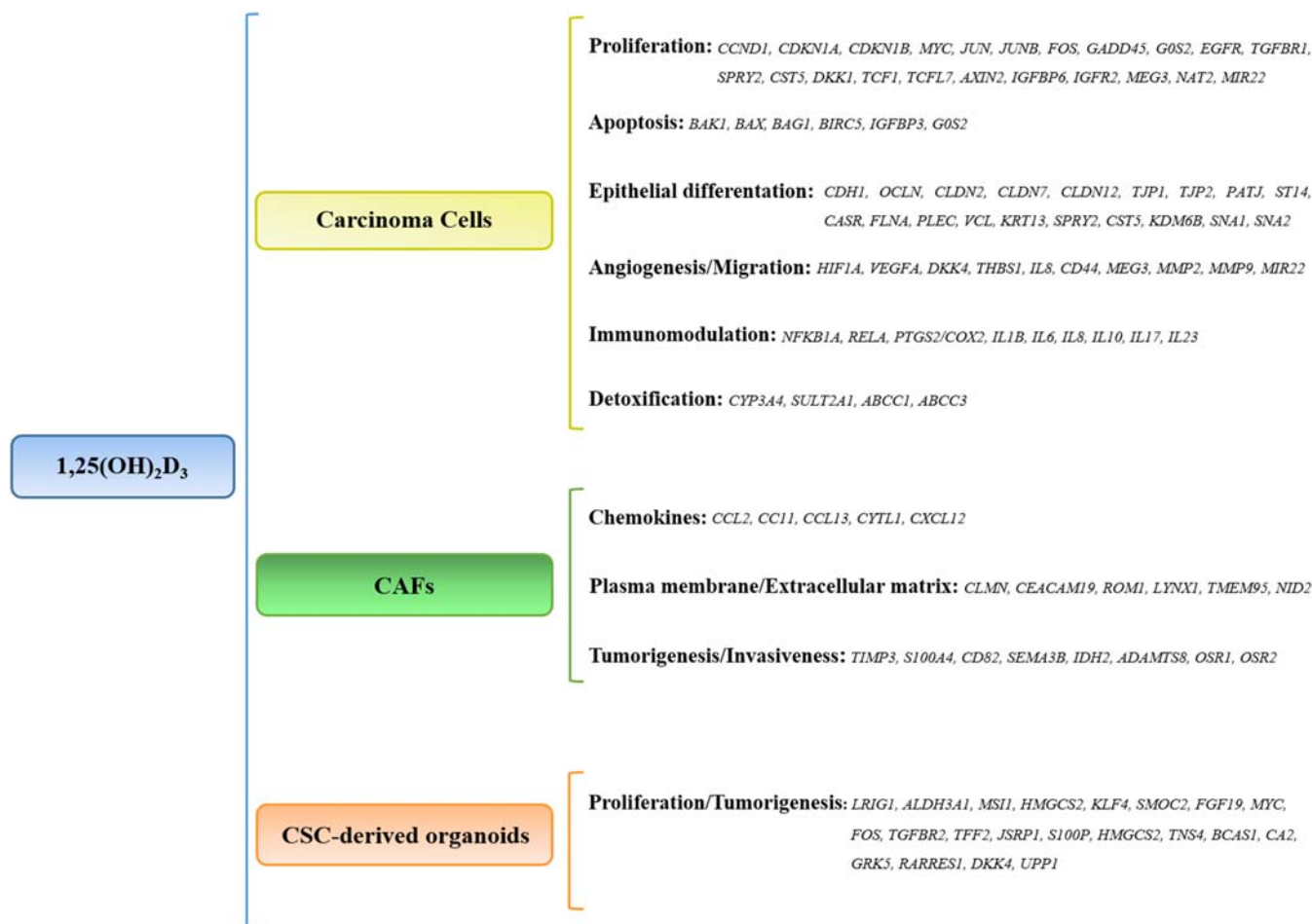
As said before, Wang and colleagues have reported that *miR-372/373* overexpression results in reduced expression of VDR RNA and protein in CRC cells [188]. This *miR-372/373* cluster is a transcriptional target of canonical Wnt signaling harboring three  $\beta$ -catenin/TCF-binding sites in its promoter region [274] and, as expected, is dysregulated in CRC probably due to aberrant activation of the pathway [275,276]. Overexpression of *miR-372/373* potentiates the stemness of CRC cells and promotes self-renewal, chemotherapy resistance, and invasive potential by upregulating stemness-related pathways (e.g., Nanog, Hedgehog) and by downregulating differentiation-associated pathways (e.g., NF- $\kappa$ B, MAPK/ERK) [188]. Altogether, these data suggest that Wnt/ $\beta$ -catenin signaling inhibits VDR expression through the induction of the *miR-372/373* cluster, which contributes to the maintenance of the cancer stem cell phenotype in CRC cells.

## 4.2 Effects on colon cancer cell proliferation, survival, and differentiation

### 4.2.1 Cell proliferation

Similar to the results found in many other cell systems, 1,25(OH)<sub>2</sub>D<sub>3</sub>, 25(OH)D<sub>3</sub>, and several less calcemic 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs inhibit the proliferation of cultured colon carcinoma cells irrespective of *TP53* status, provided that they express sufficient VDR levels [148,277]. Calcipotriol showed a similar antiproliferative effect than 1,25(OH)<sub>2</sub>D<sub>3</sub>, but more efficacy than 20-hydroxyvitamin D<sub>3</sub> [137], while EB1089 induces a higher growth arrest than 1,25(OH)<sub>2</sub>D<sub>3</sub> [278]. Recently, new low-calcemic vitamin D derivatives showed higher antiproliferative effects in HT29 cells than 1,25(OH)<sub>2</sub>D<sub>3</sub> and increased the cell growth-inhibitory effect of 5-fluorouracil (5-FU) on HT29 cells [279]. 1,25(OH)<sub>2</sub>D<sub>3</sub> induces cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> by regulating the phosphorylation of the retinoblastoma protein through the downregulation of cyclin-dependent kinases (CDKs) and the upregulation of the CDK inhibitors p21<sup>CIP</sup> and p27<sup>KIP1</sup> [280–282] (Fig. 89.2).

A major antiproliferative mechanism of 1,25(OH)<sub>2</sub>D<sub>3</sub> in colon carcinoma cells is the repression of the *MYC* oncogene, as almost all human CRCs show a *MYC*-induced transcriptional program [27]. 1,25(OH)<sub>2</sub>D<sub>3</sub> downregulates *MYC* by both direct and indirect mechanisms. As mentioned before, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits *MYC* expression directly by VDR binding to vitamin D response elements localized in its promoter and intragenic regions [261]. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits *MYC* expression indirectly by the antagonism of



**FIGURE 89.2** 1,25(OH)<sub>2</sub>D<sub>3</sub> controls a large number of genes (GeneCards symbols, [www.genecards.org](http://www.genecards.org)) in human colon carcinoma cells, CAFs, and CSC-derived organoids that are involved in crucial cancer processes.

Wnt/ $\beta$ -catenin pathway at least in part via RhoA-ROCK activation [148,221] and the induction of *CST5* gene encoding cystatin D, an inhibitor of endosomal cysteine proteases of the cathepsin family that localizes partially in the nucleus regulating several target genes [222,283]. Moreover, in other types of cancer cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> also induces the MYC antagonist MAD/MXD1 [284] and increases the interaction of FBW7 E3 ubiquitin ligase with VDR and MYC promoting MYC protein degradation [285].

Several other antiproliferative effects of vitamin D have been reported. 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of the multifunctional growth arrest and DNA damage 45 $\alpha$  (*GADD45A*) gene and *G0S2* [286–288] and regulates many other genes related to proliferation including the AP-1 components *FOS* and *JUN* [148,260,286]. In CRC cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates the expression of maternally expressed gene 3 (*MEG3*) encoding a lncRNA that has tumor-suppressive or tumor-promoting activities depending on the type of cancer [289,290]. *MEG3* inhibits CRC cell proliferation and migration by

downregulating *Clusterin*, while 1,25(OH)<sub>2</sub>D<sub>3</sub> induces direct binding of VDR to *MEG3* promoter, suggesting that *MEG3* lncRNA is involved in the antiproliferative action of 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells [291]. Moreover, vitamin D nanoemulsion (NVD) induces growth arrest at G<sub>2</sub>-phase and apoptosis and inhibits colony formation of HCT116 and HT29 CRC cells. At the protein level, treatment with NVD decreased expression of  $\beta$ -catenin, AKT, and survivin [292]. N-acetyltransferase 2 (*NAT2*) is another gene involved in the antiproliferative action of vitamin D. Thus, *NAT2* inhibits CRC cells proliferation and migration and is transcriptionally upregulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [293]. Notably, *NAT2* is repressed in CRC patients, and lower expression of *NAT2* correlates with high metastasis risk and poor patient survival.

1,25(OH)<sub>2</sub>D<sub>3</sub> also inhibits the proliferation of human colon carcinoma cells by interfering with growth factor receptor-activated pathways. Epidermal growth factor receptor (EGFR) signaling is recognized as an important player in CRC initiation and progression and is required for efficient tumorigenesis in experimental models



[294,295]. Mutual modulation between ligand-activated VDR and EGFR has been proposed in colonic tumorigenesis and in other tissues [296,297]. In CRC cells, 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces EGFR expression and promotes its ligand-induced internalization [298], and it also diminishes EGFR activity through the induction of E-cadherin expression [148,299] and the repression of *SPRY2* gene [223,300], which are, respectively, negative and positive EGFR regulators [299,301]. Besides, inhibition of the renin–angiotensin system by 1,25(OH)<sub>2</sub>D<sub>3</sub> has been proposed to mediate the antagonism of EGFR signaling in colitis-associated CRC [170]. Conversely, SNAIL1 and several cytokines may mediate the downregulation of VDR by activated EGFR [168,170].

1,25(OH)<sub>2</sub>D<sub>3</sub> interferes the insulin-like growth factor (IGF) pathway. IGF-II is overexpressed in CRC and acts as a mitogen and a survival agent [302]. In contrast, multidrug-resistant and metastatic CRC cells express low levels of IGF-binding protein (IGFBP)-6, and levels are lower in metastatic cells than in nonmetastatic cells, which suggests an antitumorigenic effect [303]. Interestingly, 1,25(OH)<sub>2</sub>D<sub>3</sub> and certain analogs inhibit IGF-II secretion and increase IGFBP-6 levels [304,305]. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> induces type II IGF receptor (IGFR-II), which accelerates IGF-II degradation and downregulates this pathway [306].

TGF- $\beta$  inhibits the growth of normal colon epithelial cells [306], and accordingly, alterations in TGF- $\beta$  signaling promote CRC cell growth, migration, invasion, angiogenesis, and metastasis. 1,25(OH)<sub>2</sub>D<sub>3</sub> induces the expression of type I TGF- $\beta$  receptor, which sensitizes CRC cells to the growth-inhibitory action of TGF- $\beta$  [286,306]. Moreover, SMAD3, a downstream protein in the TGF- $\beta$  signaling pathway, binds to SRC-1 and acts as a coactivator of VDR in the induction of 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes [307].

Another indirect mechanism of the antiproliferative effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> is the regulation of miRs. In a cohort of CRC patients, we found that *miR-22* expression is inhibited in 78% of tumor samples compared with adjacent healthy samples and is associated with CRC progression, metastasis, and relapse. Interestingly, 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates *miR-22*, and transfection of *miR-22* antagonist suppresses the antiproliferative effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> in HCT116 and SW480-ADH cells, showing that *miR-22* contributes to the antiproliferative effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> [308]. *miR-22* inhibits the proliferation and also the invasion and metastatic ability via SNAIL inhibition of CRC cells and of other cancer cell types [308–311]. Curiously, a negative feedback loop exists between *miR-22* and Sp1: while *miR-22* targets Sp1 and suppresses its activation of PTEN/AKT pathway, Sp1 binds to the promoter of *miR-22* repressing its transcription [312]. Another miRNA involved in the antiproliferative effect of vitamin D compounds is *miR-1278*,

which sensitizes CRC cells to 1,25(OH)<sub>2</sub>D<sub>3</sub> by suppressing the expression of CYP24A1 [313].

#### 4.2.2 Cell survival

The fate of cells is largely determined by the level and balance between apoptosis and autophagy. Remarkably, vitamin D has wide regulatory actions on these two processes in many cell systems.

1,25(OH)<sub>2</sub>D<sub>3</sub> plays a role in CRC cell survival by regulating genes involved in apoptosis [278,314,315] (Fig. 89.2). Thus, 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates the expression of the proapoptotic protein BAK1 and promotes redistribution of the antiapoptotic protein BAG1 from the nucleus to the cytoplasm [278,316]. Furthermore, 1,25(OH)<sub>2</sub>D<sub>3</sub> increases the level of G0S2 [286], a mitochondrial protein that interacts with BCL2 and induces apoptosis of CRC cells by preventing BCL2 from forming antiapoptotic heterodimers with BAX [317]. In a xenograft study, the inhibition of tumor growth by 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs was associated with high level of caspase-3 protein expression, which suggests that 1,25(OH)<sub>2</sub>D<sub>3</sub> induces apoptosis in malignant cells in vivo [318]. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> promotes CRC cell sensitivity to the chemotherapeutic agent 5-FU by downregulating the expression of both the antiapoptotic protein survivin and thymidylate synthase, an enzyme involved in DNA de novo synthesis that is the molecular target of 5-FU [319]. 1,25(OH)<sub>2</sub>D<sub>3</sub> restored the sensitivity of CRC cells to TRAIL-induced apoptosis by interfering with the release of IL-1 $\beta$  by macrophages in an in vitro model developed to evaluate the cross-talk between tumor-associated macrophages and CRC cells [259]. Moreover, combination of imatinib with PRI-2191, an analog of 1,25(OH)<sub>2</sub>D<sub>3</sub>, reduces stemness-related expression genes in HCT116 cells that were exposed to 5-FU more efficiently than imatinib alone, showing that this combination could be used to prevent recurrence and to compensate for vitamin D deficiency resulting from imatinib treatment [320].

The role of the *TP53* gene in the proapoptotic action of 1,25(OH)<sub>2</sub>D<sub>3</sub> is controversial. Some reports indicate that this action does not require an intact *TP53* [278,321]. However, the study by Stambolsky and colleagues [194] revealed that mutant p53 protein converts 1,25(OH)<sub>2</sub>D<sub>3</sub> into an antiapoptotic agent. This suggests that the effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on apoptosis is dependent on the *TP53* status of cancer cells. Of note, 1,25(OH)<sub>2</sub>D<sub>3</sub> and metformin have additive/synergistic antiproliferative and proapoptotic effects in CRC cell lines and other types of cells, which is modulated but not hampered by *TP53* status [322].

Vitamin D promotes cell death by autophagy in several cancer types as a protective mechanism to eliminate cells with excessive damage ([323] and refs. therein). Accordingly, vitamin D deficiency or lack of



VDR alters the expression of autophagy genes in intestinal tissues of mice and patients with IBD and dysregulates the balance between autophagy and apoptosis in small intestine, colon tissue, and mouse organoids [324,325].

#### 4.2.3 Cell differentiation

A link between the induction of differentiation and apoptosis by  $1,25(\text{OH})_2\text{D}_3$  has been suggested by the occurrence of apoptosis subsequent to the promotion of differentiation [278].  $1,25(\text{OH})_2\text{D}_3$  increases the expression and/or activity of several intestinal brush border enzymes, such as alkaline phosphatase and maltase that are widely considered as differentiation markers, and enhances the formation of microvilli [278,326–329]. It also increases the expression of a plasma membrane calcium ATPase isoform [330] that has been associated with colonic cell differentiation [331]. Moreover,  $1,25(\text{OH})_2\text{D}_3$  promotes epithelial cell differentiation through the upregulation of proteins involved in maintenance of the epithelial adhesive phenotype such as occludin, zonula occludens (ZO)-1 and -2, and claudin-2, -7, and -12 of tight junctions, E-cadherin of adherens junctions, and plectin of hemidesmosomes [224,286,332] (Fig. 89.2). A key action seems to be the induction of *CDH1*/E-cadherin gene transcription, which relies on a rapid signaling pathway triggered by the entry to the cytoplasm of  $\text{Ca}^{2+}$  from the external medium and the subsequent activation of a RhoA-ROCK1-MSK1/2 cascade [148,221]. Given the repressive effect of *SPRY2* on these intercellular adhesion genes and also on regulators of the polarized epithelial phenotype such as *LLGL2*, *PATJ*, and *ST14* [223,300], the downregulation of *SPRY2* seems to be a major contributor to the prodifferentiation action of  $1,25(\text{OH})_2\text{D}_3$ . Another mechanism by which  $1,25(\text{OH})_2\text{D}_3$  promotes epithelial differentiation is the antagonism of the major EMT inducers *SNAIL* and *SNAIL2* genes, probably as a consequence of the cross-inhibition of the Wnt/ $\beta$ -catenin, TGF- $\beta$ , and EGF pathways ([333] and refs therein).

$1,25(\text{OH})_2\text{D}_3$  also increases the expression of proteins associated with the actin cytoskeleton and intermediate filaments such as keratin-13, vinculin, and filamin A [148,286]. Filamin A has been implicated in cell motility, adhesion, and invasion [334] and in the regulation of nuclear shape during EMT and DNA double-strand break repair [335,336]. Also, it has been reported that low levels of filamin A are found in CRC and that this downregulation is associated with poor clinical prognosis [337].  $1,25(\text{OH})_2\text{D}_3$  can also induce the calcium sensing receptor (CaSR), a G protein-coupled receptor with a key role in regulating calcium homeostasis, and the proliferation and differentiation of normal colonic epithelium and CRC cells [338–342].

$1,25(\text{OH})_2\text{D}_3$  attenuates the effects of TGF- $\beta_1/\beta_2$  on SW480 and HT29 cells, which include an increase in invasion and migration, expression of EMT-related transcription factors and F-actin, and secretion of matrix metalloproteinases (MMP)-2 and -9 [343].

In summary, there are several mechanisms by which  $1,25(\text{OH})_2\text{D}_3$  inhibits the EMT process, which contributes to the promotion of epithelial cell differentiation [344].

### 4.3 Antiangiogenic, antiinflammatory, immunomodulatory, and detoxifying effects

#### 4.3.1 Angiogenesis

The role of  $1,25(\text{OH})_2\text{D}_3$  on the angiogenic capacity of CRC cells relies to a great extent on its ability to suppress the expression and activity of hypoxia-inducible factor (HIF)- $1\alpha$ , a key transcription factor in hypoxia-induced angiogenesis [345]. In a study using prostate cancer cell xenografts,  $1,25(\text{OH})_2\text{D}_3$  decreased the proliferation of tumor-derived endothelial cells in wild-type but not in *Vdr*<sup>-/-</sup> mice, which showed increased vasculature and higher Hif- $1\alpha$  and vascular endothelial growth factor (VEGF) levels [346] (Fig. 89.2). Also, in colitis animal models, vitamin D suppresses HIF- $1\alpha$  overexpression in colonic epithelial cells through the inhibition of NF- $\kappa\text{B}$  pathway [347]. In SW480-ADH CRC cells,  $1,25(\text{OH})_2\text{D}_3$  regulates the expression of VEGF-A and thrombospondin-1, two major opposing factors that control tumor angiogenesis [348]. In addition, prolonged administration of  $1,25(\text{OH})_2\text{D}_3$  to Wistar rats inhibits the development of azoxymethane (AOM)-induced colon tumors in association with a decrease in VEGF expression and vessel counts [349].  $1,25(\text{OH})_2\text{D}_3$  strongly represses DKK4, a weak Wnt antagonist that promotes invasion and angiogenesis in cultured CRC cells and is upregulated in human CRC [241].

#### 4.3.2 Inflammation and immunomodulation

Chronic inflammation is associated with abnormal immune response and may be caused by a variety of factors including bacterial and viral infections and chemical irritants. A considerable amount of evidence suggests that chronic inflammation can predispose to cancer. In particular, the association between chronic IBDs and the increased risk of CRC is well documented [5,350]. Common events in the inflammatory process such as inflammatory cell infiltration and increased expression of proinflammatory cytokines seem to be involved in sporadic and heritable CRC [350]. An antiinflammatory action of vitamin D and a link between vitamin D deficiency and the incidence of IBD have been proposed. The mechanisms by which vitamin D affects inflammation-related CRC include the inhibition of

NF- $\kappa$ B pathway, prostaglandin (PG)-endoperoxide synthase (PTGS-2, also known as COX-2), and several cytokines (Fig. 89.2).

Patients with UC or CD show reduced levels of VDR in intestinal epithelium. Likewise, overexpression of Vdr in experimental models inhibits the colitis-associated increase in proinflammatory cytokines such as TNF, IL-1, and CCL2 and protects mice from developing colitis [351]. In addition, Ananthakrishnan and colleagues proposed that higher predicted plasma 25(OH)D levels significantly reduce the risk of CD [352]. In a study of two cohorts including 798 CRC patients, a high level of circulating 25(OH)D<sub>3</sub> is inversely associated with IL-6 level at diagnosis and with the summary inflammatory *z*-score at diagnosis, at 6 months after diagnosis and over the course of 2 years. Moreover, 25(OH)D<sub>3</sub> level was inversely associated with that of IL-10 over this period [353]. In another study with 117 CRC patients, serum 25(OH)D levels inversely correlated with several systemic inflammatory markers such as blood neutrophil count, serum C reactive protein, blood neutrophil/lymphocyte ratio, and serum IL-6 levels, but not with diagnosis, while the lowest serum levels associated with mismatch repair deficiency, serrate morphology, and high body mass index [354].

A definitive demonstration of a protective role of vitamin D compounds in IBD patients has not been fully demonstrated, as some studies on the effect of vitamin D supplementation on inflammatory markers in patients with colorectal adenomas showed inconclusive results. Thus, the generation of intestine-specific and myeloid-specific *Vdr*<sup>-/-</sup> mice in a dextran sulfate sodium (DSS) model of experimental colitis showed that *Vdr* inactivation in macrophages and granulocytes mildly affected colitis-associated symptoms and increased proinflammatory cytokines and  $\beta$ -defensin-1 in colon *descendens* of mice with colitis and in inflamed colon [355]. On the contrary, loss of *Vdr* in the intestine did not change colitis-associated symptoms or mucosal cytokine expression. Hodge and colleagues have reported that 1-year treatment with vitamin D (1000 IU/d) and calcium (1.2 mg/d) alone or in combination failed to affect the expression in the rectal mucosa of TLR-4/5 and phospho-IKK $\alpha$ / $\beta$ , two biomarkers of inflammation, in a subset of 105 participants from a colon adenoma recurrence chemoprevention clinical trial [356]. Moreover, Protiva and colleagues showed that supplementation of a Western diet with 1,25(OH)<sub>2</sub>D<sub>3</sub> increased the expression of genes involved in inflammation in human colon, an effect that was reversed by calcium supplementation [357].

VDR is expressed by almost all cell types of the immune system, and accordingly, a large number of studies support a key immunomodulatory role of 1,25(OH)<sub>2</sub>D<sub>3</sub> [358–360]. Many times, 1,25(OH)<sub>2</sub>D<sub>3</sub> has

been considered an enhancer of the innate immune system against infections and tumor cells by activating the responsive cells (macrophages, neutrophils), and a repressor of the adaptive immune reactions by deactivating dendritic cells and CD4<sup>+</sup> type 1 helper T (Th1) cell responses (production of interferon- $\gamma$ , IL-1, IL-6, IL-12) and promoting Th2 and Treg cell responses (production of IL-10, IL-4, IL-5), which, in contrast, could potentially reduce the protection against tumors. However, this inhibitory effect on the proinflammatory Th1 response has been observed in experimental settings following overstimulation of the cells. Moreover, the long-term consideration that the induction of IL-10 by VDR ligands has only protumoral consequences is unclear, as IL-10 seems to have a dual effect in cancer also promoting tumor-specific T cell immune surveillance and hindering pathogenic inflammation [361]. Thus, in line with its selection during evolution, vitamin D has probably the essential action of a regulator, which is potentiating the early defensive proinflammatory activity of the immune system against infections and tumors and inhibiting at later stages the overactivation and self-reactivity to prevent adverse consequences such as chronic inflammation or autoimmunity [362–364].

Gut epithelial VDR is important to protect the intestinal barrier integrity and regulates the gut innate immunity. In mice, mucosal inflammation is controlled by gut epithelial Vdr signaling by suppressing epithelial cell apoptosis [365]. 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling is required for colonic group 3 innate lymphoid cells (ILC3)–mediated innate immunity against *Citrobacter rodentium* (*C. rodentium*) through regulation of cell proliferation [366]. Besides, mice with depleted Vdr expression show a more severe clinical colitis and stronger Th1 and Th7 responses than control mice, with enhanced mucosal inflammatory responses in the gut.

COX-2 synthesizes PGs from arachidonic acid and other fatty acids. PGs are mediators of inflammatory processes and regulate the proliferation, apoptosis, angiogenesis, and other parameters of several types of cancer cells [367,368]. Moreover, PGs, such as PGE<sub>2</sub>, stimulate CRC cell proliferation, and COX-2 is overexpressed in CRC patients [369]. A large number of epidemiological studies using aspirin and other COX inhibitors support the benefit of COX-2 inhibition for CRC patients [367,370]. Although 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces the protumorigenic effect of PGE<sub>2</sub> in prostate cancer cells by inhibiting COX-2 and decreasing levels of PGE<sub>2</sub> and two PG receptors (EP2 and FP) [371], similar effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells have not been clearly established. In chemically induced CRC models, treatment with vitamin D or some analogs reduced COX-2 expression [372,373]. An indirect mechanism to control the production of PGE<sub>2</sub> by 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells

could be mediated by the activation of CaSR, as this inhibits phospholipase A2 activity and thus reduces the amount of arachidonic acid available for PG synthesis [374]. Importantly, vitamin D and calcium favorably modulate the balance of expression of COX-2 and 15-hydroxyPG dehydrogenase, its physiological antagonist, in the normal-appearing colorectal mucosa of patients with colorectal adenoma [375].

NF- $\kappa$ B is a protein complex that controls the transcription of many genes, including a large number of those responsible for cytokine production and cell survival. NF- $\kappa$ B is present in all cell types and is a key regulator of immune responses and inflammation. Improper regulation of NF- $\kappa$ B has been linked to cancer, inflammatory, and autoimmune diseases [367,376]. In unstimulated cells, NF- $\kappa$ B is in an inactive state sequestered in the cytoplasm by a family of inhibitors, called inhibitors of  $\kappa$ B (I $\kappa$ B). Upon stimulation by proinflammatory cytokines, NF- $\kappa$ B translocates from the cytosol to the cell nucleus, where it stimulates the transcription of genes involved in proinflammatory processes, such as IL-6, IL-8, TNF- $\alpha$ , and COX-2 [367]. In CRC cells, NF- $\kappa$ B activity is inhibited by 1,25(OH) $_2$ D $_3$  through different mechanisms, including inactivation of the p65 subunit of the NF- $\kappa$ B complex, thus preventing the binding of NF- $\kappa$ B to DNA [377], and increasing the expression of the inhibitor subunit I $\kappa$ B [376–379]. Moreover, CRC cells treated with an antagonist of VDR showed increased NF- $\kappa$ B activity and reduced I $\kappa$ B expression [380]. Accordingly, high dietary vitamin D decreases NF- $\kappa$ B activation, and thereby reduces inflammation in mouse colonic epithelial cells [381], and *Vdr* $^{-/-}$  mice exhibit a proinflammatory phenotype associated with increased NF- $\kappa$ B activity in the intestine [382]. Thus, 1,25(OH) $_2$ D $_3$  exerts antiinflammatory properties and inhibits IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , and possibly other cytokines (for example, IL-17, IL-12, and TGF- $\beta$ ), which are expressed at high levels in CRC patients and may be involved in CRC progression [383–385]. This effect of 1,25(OH) $_2$ D $_3$  relies at least in part on the blockade of NF- $\kappa$ B [376–379].

In contrast to the antiinflammatory and immunomodulatory role proposed for vitamin D in CRC, it has been suggested that low levels of 25(OH)D or low VDR expression in CRC patients might be a consequence of chronic inflammation or of infection rather than the cause [386]. Some studies support this reverse causality idea, showing that the presence of proinflammatory cytokines might impair 1,25(OH) $_2$ D $_3$  synthesis limiting its antiinflammatory action in CRC cells [387]. Probably, mutual regulation exists between vitamin D status and inflammation, and the result in each situation will depend on the relative intensity of the respective signals. Supporting this idea, the transcriptional response to vitamin D is impaired in COVID-19 patients who have

a strong proinflammatory Th1 cell phenotype, which leads to a failure to switch to IL-10 production and inflammatory resolution [388].

In summary, there is overwhelming evidence from experimental in vitro and in vivo studies indicating a homeostatic role of vitamin D on the biology of many types of cells and reactions of the immune system in a large series of systems and contexts including the protection against CRC. As said by Martens and colleagues in an excellent review, although the translation of these observations to the clinic is still pending, it seems clear the convenience of avoiding vitamin D deficiency to maintain a fully functional immune system [364].

#### 4.3.3 Microbiome

An increasing interest in the role of the microbiome in human health and disease including CRC has boosted the number of studies on vitamin D and the gut microbiome [74,389–391]. A mutual interaction exists between vitamin D and the intestinal microbiome that is connected to CRC. Thus, there is a link between alteration of the gut microbiome, known as dysbiosis, and CRC. Vitamin D affects the gut microbiota composition and activity as shown by the effect of vitamin D deficiency promoting gut permeability, colon mucosa bacterial infiltration, and translocation of intestinal pathogens leading to gut inflammation [390–392]. As bacteria lack VDR, the effect of vitamin D is mediated by the host, to a great extent via the regulation of the epithelial barrier and immune system.

*Fusobacterium nucleatum* (Fn) has been associated with CRC. *FadA*, a virulence factor identified from Fn, binds E-cadherin, activates  $\beta$ -catenin signaling, and regulates the inflammatory and oncogenic responses [393]. *Vdr* $^{-/-}$  mice develop dysbiosis and display lower expression of E-cadherin in gut epithelial and immune cells, fewer tolerogenic dendritic cells, and intensified gut inflammation compared with wild-type (wt) mice, leading to higher susceptibility to damage in the gut [394]. In line with this, vitamin D-deficient mice that were given 2% of DSS displayed a pronounced epithelial barrier dysfunction, were more susceptible to adherent-invasive *Escherichia coli* colonization, and showed aggravated colonic injury [395]. Also, in agreement, fecal samples from *Vdr* $^{-/-}$  mice revealed changes in fecal microbiomes showing that *Vdr* affects different pathways such as nucleotide-binding oligomerization domain-like receptor, signal transduction, detoxification, amino acid synthesis, infections, cancer, and other diseases [396]. *Vdr* $^{-/-}$  mice had also fewer *C. rodentium* in the feces and more I1-22 producing innate lymphoid cells, and less antibacterial peptides than wt mice. Treatment of *Vdr* $^{-/-}$  mice with antibiotics reversed colonization resistance to *C. rodentium* infection, and transferring the microbiota of *Vdr* $^{-/-}$  to wt germ-free mice



resulted in colonization resistance [397]. Moreover, lower level of vitamin D in CD1 mice reduced the expression of Vdr and increased that of proinflammatory genes in the colon, and it causes lower numbers of colonic *Bacterioides/Prevotella* and higher serum lipopolysaccharides (LPS) concentration [398]. Furthermore, prebiotics but not probiotics affect the transformation of 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) to 2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-f]quinoline-7-one (7-OH-IQ) by fecal microbiota [399], showing that microbiota can also regulate the response to carcinogens [400]. Notably, while regulation of the Vdr is a common mechanism used in the host defense against pathogens, some microorganisms such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Aspergillus fumigatus*, Epstein–Barr virus, and HIV have been shown to decrease innate immune defense by repressing Vdr [386].

Several studies support these findings. He and colleagues reported that gut epithelial Vdr regulates microbiome-dependent mucosal inflammation by suppressing intestinal cell apoptosis [365]. In an experimental model of IBD, Cantorna and colleagues showed that vitamin D–deficient mice presented a reduced frequency of FoxP3<sup>+</sup> and RorγT/FoxP3<sup>+</sup> Treg cell population in the colon. Germ-free recipients of vitamin D–sufficient mice microbiota had more RorγT/FoxP3<sup>+</sup> Treg cells than those of vitamin D-deficient mice microbiota. Higher frequency of the RorγT/FoxP3<sup>+</sup> Treg cell population in vitamin D sufficiency colon correlated with higher amount of *Clostridium XIVa* and *Bacteroides* in vitamin D–sufficient compared with vitamin D–deficient cecum. Also, vitamin D–deficient mice with fewer RorγT/FoxP3<sup>+</sup> Treg cells were more susceptible to colitis than vitamin D–sufficient mice. The authors indicated that early vitamin D status modulates the microbiota to optimize the population of colonic RorγT/FoxP3<sup>+</sup> Treg cells that are important for resistance to colitis [401]. In a randomized clinical trial, no differences were found in microbiome α-diversity between vitamin D and placebo groups at baseline and follow-up on the effect of vitamin D on fecal microbiota of overweight or obese otherwise healthy adults. However, there was an association between community composition and vitamin D supplementation at the genus level (*Lachnospira*, *Blautia*, *Coproccoccus*, *Ruminococcus*), indicating effects on fecal microbiota [402].

A model of AOM/DSS-induced CRC in intestinal conditional *Vdr*<sup>−/−</sup> (*Vdr*<sup>ΔIEC</sup>) mice showed that *Vdr* deficiency changes bacterial profile from normal to carcinogenesis susceptibility. Fecal samples from this mice model produced an activation of STAT3 signaling in human and mouse organoids. Vdr protein bound to the *Jak2* promoter, while lack of Vdr increased *Jak2* function in response to intestinal dysbiosis, and the microbiome-

induced activation of Stat3 was diminished by an inhibitor of Jak/Stat [403]. Moreover, HCT116 cells treated with conditioned medium (CM) from probiotic lactic acid bacteria (LAB) showed increased expression of VDR and of its target *CAMP* gene encoding cathelicidin. Two probiotic proteins, P40 and P75, present in the LAB-CM increased VDR in organoids and protected them from the inflammatory response induce by TNF-α [404]. Importantly, genome-wide association analysis of the gut microbiome in two large cohorts of individuals has identified VDR as a factor influencing the gut microbiota [405].

#### 4.3.4 Detoxification

The gastrointestinal tract and liver are the main organs responsible for drug metabolism. They play key roles in the defense against toxins and other xenobiotics in the diet that may contribute to CRC development [406]. The biotransformation process, often referred to as a detoxification process, involves two distinct stages. Phase I reactions include the introduction of polar functional groups into the molecule or modification by oxidation, reduction, or hydrolysis. Phase II reactions comprise chemical conjugations to water-soluble molecules. These reactions are carried out by a large number of enzymes, including the CYP450 family and sulfotransferases among others.

1,25(OH)<sub>2</sub>D<sub>3</sub> has an impact on the detoxification process by regulating the expression of some of these enzymes in the intestine [407] (Fig. 89.2). The best example is CYP3A4, which is considered the most important human drug-metabolizing enzyme and is induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells [408]. In addition, SULT2A1 (a phase II sulfotransferase) and members of the multidrug resistance–associated protein (MRP) family are also induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> in CRC cells [409,410]. Recently, SULT2A1 showed sulfating activity on all vitamin D<sub>3</sub>–related compounds, SULT1A1 just for 1,25(OH)<sub>2</sub>D<sub>3</sub> and SULT2B1a/SULT2B1b for 7-dehydrocholesterol [411]. However, SULT2B1b overexpression in CRC promotes cell growth and invasion in vitro, and accordingly, high SULT2B1b is an unfavorable prognosis indicator [412].

CYP3A4, SULT2A1, and MRP3 are involved in the elimination of the secondary bile acid LCA, which induces DNA damage and inhibits DNA repair enzymes in colonic cells. Accordingly, LCA promotes CRC in experimental animals, and high levels of LCA have been found in CRC patients [413]. Since VDR functions as a receptor for LCA [414] and the activation of VDR by LCA induces CYP3A4, SULT2A1, and MRP3 expression, this is a feedback mechanism that results in LCA elimination in the colon. Thus, VDR helps to decrease the level of its agonist, the deleterious secondary bile acid LCA.



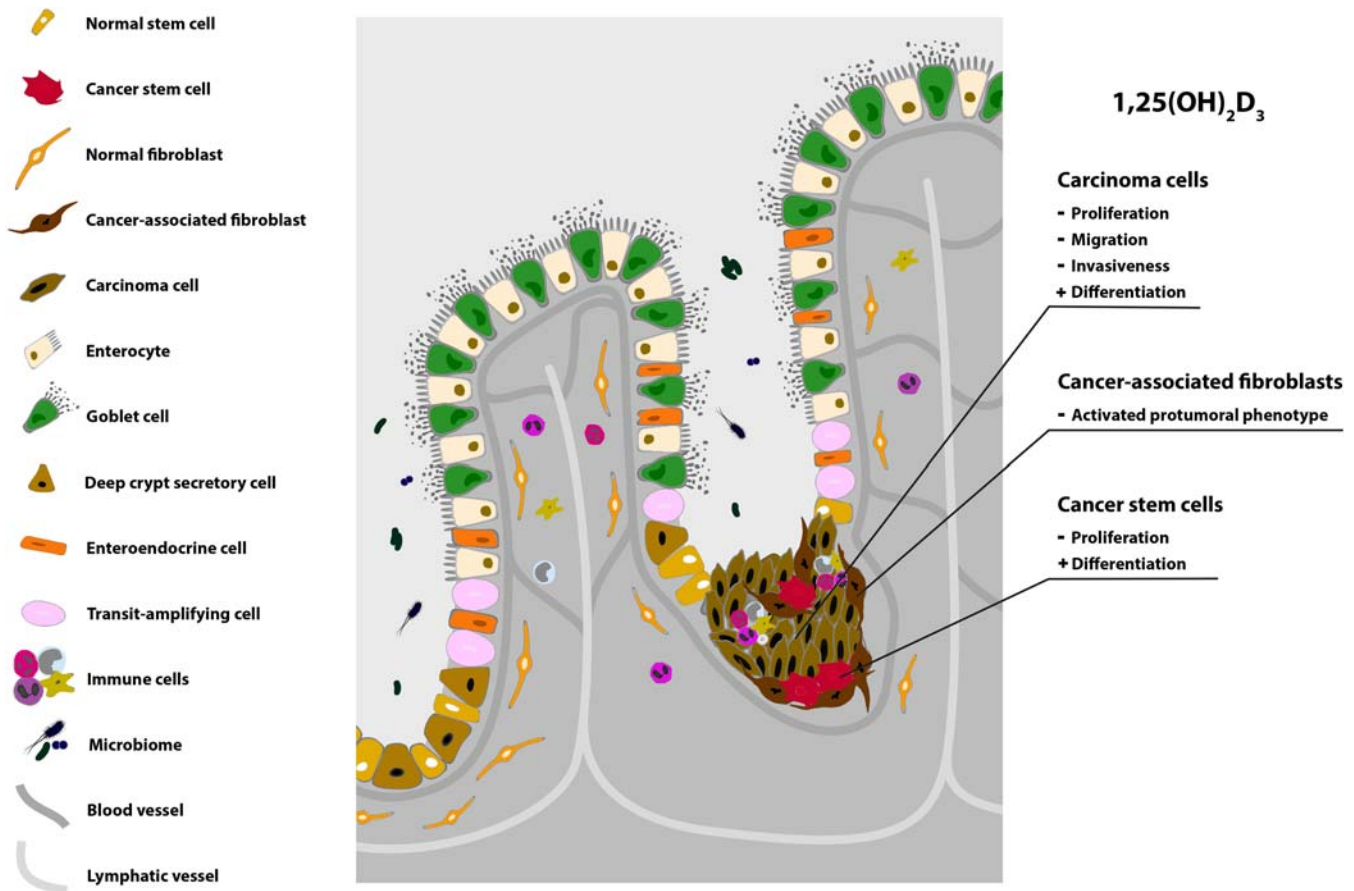
Interestingly, a positive correlation between levels of 25(OH)D<sub>3</sub> and total thiol concentration (TTC), a biomarker of antioxidant capacity, has been reported. Thus, there is an inverse association between 25(OH)D and mortality in patients with low or medium TTC, suggesting that survival advantages in CRC patients with adequate vitamin D depend on antioxidant capacity and are most pronounced in cases of low antioxidant capacity [85].

#### 4.4 Effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in colorectal cancer—associated fibroblasts

Tumor progression depends not only on tumor cells but also on tumor microenvironment or stroma, which comprehends the ECM, surrounding and infiltrating host (stromal) cells in the tumor mass and secreted factors. The interaction of tumor cells with these stromal constituents is crucial at all stages of tumor development [415]. Stromal fibroblasts or CAFs are the cell type most abundant in tumor microenvironment, and a mutual dynamic interaction exists between tumor cells and CAFs [416]. CAFs are heterogeneous, as they can be originated by the phenotypic change (activation) of resident fibroblasts, by the recruitment and activation of bone marrow-derived fibrocytes and mesenchymal stem cells, or by the transdifferentiation of epithelial, endothelial, or smooth muscle cells, adipocytes, or pericytes in response to signals secreted by tumor cells or by other cells of the tumor stroma. Upon recruitment and activation, CAFs can remodel the ECM (volume, composition, and organization), secrete soluble factors that act in a paracrine manner on carcinoma and immune cells, modify tumor metabolism and response to therapy, and induce the recruitment of bone marrow precursors [417]. Thus, CAFs are thought to actively contribute to the tumorigenic process promoting cancer invasion, angiogenesis, and metastasis and inhibiting the immune response [418]. In CRC, CAFs also increase the frequency of tumor-initiating cells [16]. In addition, tumor stroma can function as a barrier that prevents efficient intratumor drug delivery. Accordingly, poor prognosis CRC subtypes are characterized by a prominent desmoplastic reaction and elevated expression of a CAFs-associated gene signature [15,16,419]. These findings led to the idea that combination therapies that target both the stromal and the neoplastic cells may provide therapeutic benefits for cancer patients [418]. However, the role of stromal fibroblasts in tumor progression is complex, as tumor-suppressive effects of these cells have also been described. Thus, treatments directed at reprogramming activated stromal fibroblasts to nonactivated/normal stromal cells may be more effective than

those focused on general depletion of the tumor-associated stroma [418,420–422].

Our group studied the possible effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on CRC stromal fibroblasts. We established primary cultures of colon normal fibroblasts (NFs) and CAFs, derived from fresh biopsies of CRC patients, and we found that all the primary cultures responded to 1,25(OH)<sub>2</sub>D<sub>3</sub> and expressed VDR. Besides, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the protumoral action of activated fibroblasts. First, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the capacity of alter the ECM, tested as its capacity to remodel collagen gels. And second, 1,25(OH)<sub>2</sub>D<sub>3</sub> reduced the ability of CAFs to promote the migration of CRC cells. This showed that 1,25(OH)<sub>2</sub>D<sub>3</sub> reprograms CAFs to a less activated phenotype. VDR expression in these primary cultures correlates with the inhibitory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the promigratory effect of fibroblasts on CRC cells, suggesting that this antitumoral action of 1,25(OH)<sub>2</sub>D<sub>3</sub> depends on VDR expression in colon fibroblasts [160]. By microarray analyses, we observed the changes that 1,25(OH)<sub>2</sub>D<sub>3</sub> provokes in the gene expression profile, regulating more than 1000 genes in NFs and CAFs, with a 21% overlap. 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes are involved in cell adhesion, differentiation and migration, tissue remodeling, blood vessel development, and inflammatory response and encoded mainly for ECM components and cytokines (Fig. 89.2). Moreover, we identified a gene signature imposed by 1,25(OH)<sub>2</sub>D<sub>3</sub>, which correlates with a better prognosis of CRC patients from publicly available data sets, showing the clinical relevance of the gene expression effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in CAFs. Additionally, we studied the VDR expression in a cohort of 658 metastatic CRC patients with prolonged clinical follow-up. High VDR expression in carcinoma cells or in tumor fibroblasts, and particularly in both, was associated with a better OS and disease-free survival independently of its expression in carcinoma cells [160]. Also, multivariate Cox regression analysis showed that VDR expression in tumor stromal fibroblasts is an independent predictor of OS in CRC. Interestingly, the regulation by 1,25(OH)<sub>2</sub>D<sub>3</sub> of CAFs genes encoding cytokines and growth factors suggests that it may also affect in a paracrine manner the biology of carcinoma cells and other cell types in the tumor microenvironment. Accordingly, 1,25(OH)<sub>2</sub>D<sub>3</sub> decreases the amount of *miR-10a-5p* found in the exosomes secreted by human pancreatic CAFs, which attenuates the promigratory and proinvasive effects that these CAFs exert on pancreatic carcinoma cells [423]. Together, all these data indicate that CAFs from CRC patients express VDR and 1,25(OH)<sub>2</sub>D<sub>3</sub> regulates its gene expression, biology, and phenotype. Thus, the anticancer action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on CRC is mediated by its direct action on CRC cells, and also by its modulation of the tumorigenic properties



**FIGURE 89.3**  $1,25(\text{OH})_2\text{D}_3$  protects against CRC acting on carcinoma cells, cancer-associated fibroblasts, and cancer stem cells. It inhibits the proliferation, migration, and invasion capacities of colon carcinoma cells while promoting their differentiated phenotype in part through the interference of the Wnt/ $\beta$ -catenin signaling pathway.  $1,25(\text{OH})_2\text{D}_3$  sensitizes these cells to apoptotic signals and reduces their angiogenic potential, modulates several immune responses, reduces the dysbiosis, and contributes to clear mutagens and xenobiotics. In addition,  $1,25(\text{OH})_2\text{D}_3$  inhibits the protumorigenic effects (migration, invasion) of cancer-associated fibroblasts on carcinoma cells and perhaps other stromal cell types, and acts on cancer stem cells attenuating their proliferation and promoting some differentiation traits.

of CAFs (Fig. 89.3). These results support the possible use of VDR agonist as antistromal agents in CRC and suggest that they may benefit patients even if their carcinoma cells lack VDR provided that CAFs express sufficient VDR level.

Due to the importance of the interference of Wnt/ $\beta$ -catenin signaling by  $1,25(\text{OH})_2\text{D}_3$  in carcinoma cells, we investigated their possible interaction in colon fibroblasts. We chose Wnt3A as a representative Wnt protein since it is one of the best described canonical Wnts. Global transcriptomic studies by RNA-seq analysis of the action of single and combined  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A treatments of the CCD-18Co human colon myofibroblast cell line showed that both agents have additive gene regulatory effects [424]. A higher number of genes were found regulated by the combination of  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A than by the single treatments: 800 genes were common targets of both agents, while 1309 genes were significantly regulated by the

combined treatment but not by the single treatments [424]. The expected additive effect explained 83% of the variability observed in the combined treatment, suggesting that most of these genes are also regulated by the single treatments. Thus, the results of the combined treatment were largely predicted by the sum of the effects of the two single treatments. We also studied the effects of  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A to modulate the phenotype of CCD-18Co myofibroblasts. Regarding their ability to remodel the ECM, we found that while  $1,25(\text{OH})_2\text{D}_3$  inhibited the capacity of CCD-18Co cells to contract collagen gels, Wnt3A had the opposite effect, and the combined treatment reduced the effect of each single treatment. Both  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A reduced the proliferation of CCD-18Co myofibroblasts, and the combined treatment showed an additive effect. Additionally,  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A single treatments inhibited the migration of CCD-18Co cells and IMR90 human lung fibroblasts, and their combination had a

similar effect than the individual treatments [424]. These studies were expanded to patient-derived primary NFs and CAFs. We analyzed in five paired NFs and CAFs cultures the expression of three genes identified in the RNA-seq study (*OSR2*, *OSR1*, and *PGD*) carried out in the CCD-18Co myofibroblasts.  $1,25(\text{OH})_2\text{D}_3$  regulated all these genes in NFs and CAFs except for *PGD* in CAFs that was induced by  $1,25(\text{OH})_2\text{D}_3$  in four out of five primary cultures but did not reach statistical significance. Moreover,  $1,25(\text{OH})_2\text{D}_3$  significantly inhibited the capacity of NFs to contract collagen gels, while the effect of Wnt3A was variable, increasing the contraction of the gels in four patients but having an opposite effect in one. The combined effects of  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A had an intermediate effect between both single treatments [424]. In conclusion,  $1,25(\text{OH})_2\text{D}_3$  and Wnt3A are strong regulators of the gene expression and phenotype of colon fibroblasts. However, in contrast to the antagonism reported in carcinoma cells, they have an additive and partially overlapping effect in fibroblasts [424,425].

Reduced VDR protein is associated with an increased migration of fibroblasts in CD, and treatment with  $1,25(\text{OH})_2\text{D}_3$  led to VDR upregulation and prevented the increase in fibroblast migration. Accordingly, lower collagen deposition and a preserved histological architecture was found in grafts obtained from vitamin D-treated mice compared with vehicle-treated mice [426]. The collagen layer was thicker in vehicle-treated mice than in vitamin D-treated mice, which showed reduced levels of *Col1a1* RNA and protein and of vimentin protein. Vitamin D-treated mice had also reduced *Cd86* and *Il-6* expression [426]. These data suggest that vitamin D reduces murine intestinal fibrosis.

#### 4.5 Effects of $1,25(\text{OH})_2\text{D}_3$ in colorectal stem cells—derived organoids

The Cancer Stem Cell (CSC) theory proposes that tumors initiate, progress, and perhaps become resistant to therapies due to the mutation of tissue resident stem cells (SCs). Intestinal SCs (ISCs) reside at the crypt bottom, and presently the expression at the plasma membrane of *Lgr5* protein is considered their best marker [427]. In concordance with the CSC theory, the deletion of *Apc* gene in *Lgr5*<sup>+</sup> ISC, but not in partially or fully differentiated intestinal cells, leads to the formation of adenomas in mice [428].

These data have prompted studies on the putative effect of vitamin D compounds on SCs and CSCs of several tissues and neoplasias. To this aim, our group faced the analysis of  $1,25(\text{OH})_2\text{D}_3$  action on human colorectal CSC by establishing organoid cultures from patient-derived matched normal and tumor tissue.

Organoids are three-dimensional (3D), self-organized multicellular structures generated by stem cells embedded in an ECM that is covered by a complex tissue-specific serum-free medium containing a mixture of growth factors, vitamins, agents, and inhibitors [429,430]. Although with a series of limitations, organoids recapitulate some of the features of a particular organ or tumor of origin and thus, are today considered a better system to study cancer processes and the activity of agents and drugs than immortalized cell line monolayers grown for decades on plastic dishes and animal models [431–433].

Human colon organoids have been used to study genome wide effects of  $1,25(\text{OH})_2\text{D}_3$ . By RNA-seq and chromatin immunoprecipitation-sequencing (ChIP-seq) assays, our group has shown that  $1,25(\text{OH})_2\text{D}_3$  profoundly and differentially regulates the gene expression profile of six CRC patient-derived matched normal and tumor organoid cultures. In normal organoids, 96 h treatment with  $1,25(\text{OH})_2\text{D}_3$  induced stemness-related genes (*LGR5*, *SMOC2*, *LRIG1*, *MSI1*, *PTK7*, and *MEX3A*), some of which (*SMOC2*, *MSI1*) were identified as direct targets [434]. By contrast, in tumor organoids,  $1,25(\text{OH})_2\text{D}_3$  had only minor effects on stemness genes. Notably, in both normal and tumor organoids,  $1,25(\text{OH})_2\text{D}_3$  reduced cell proliferation and the expression of proliferation and tumorigenesis genes such as *GRK5*, *RARRES1*, *S100P*, *TNS4*, *ALDH3A1*, *BCAS1*, *S100P*, and *SERPINE1* without affecting Wnt target genes, which agrees with the key role of Wnt and Notch pathways as main responsible for intestinal crypt SC stemness (Fig. 89.2). Of note, an exception is the down-regulation by  $1,25(\text{OH})_2\text{D}_3$  in both normal and tumor organoids of *DKK4*, a Wnt target that as said before encodes an inhibitor of Wnt/ $\beta$ -catenin signaling up-regulated in CRC and other neoplasias that has tumor-promoting effects including the induction of chemotherapy resistance [241,244,245,435]. In line with this, gene set enrichment analysis (GSEA) showed an inverse association of the  $1,25(\text{OH})_2\text{D}_3$  profile (RNA-seq) with E2F, mTOR, and MYC proliferative signatures and, contrarily, a significant enrichment in  $1,25(\text{OH})_2\text{D}_3$ -treated tumor organoids of the differentiation signature EPHB2<sup>low</sup> versus EPHB2<sup>high</sup> of human colon cells [434]. Remarkably, electron microscopy analyses revealed the induction by  $1,25(\text{OH})_2\text{D}_3$  of several cell differentiation traits in tumor organoids such as cell-to-cell adhesion structures, chromatin decondensation, and increased cytoplasmic organelle. In another study, we compared the transcriptomic profiles of both types of organoids from colon and rectum, and also found an induction of stemness genes by  $1,25(\text{OH})_2\text{D}_3$  in normal but not in tumor rectum organoids [436]. Together, these data suggest an antitumor action of  $1,25(\text{OH})_2\text{D}_3$  on CRC acting on CSC (Fig. 89.3).



In a later study, Li and colleagues reported the effect of  $1,25(\text{OH})_2\text{D}_3$  on the colon organoids of one healthy individual [437]. The authors used assay for transposase-accessible chromatin sequencing (ATAC-seq) to analyze chromatin accessibility at 4 and 8 h of  $1,25(\text{OH})_2\text{D}_3$  treatment and RNA-seq to assess the transcriptional response to  $1,25(\text{OH})_2\text{D}_3$  at 6 and 24 h posttreatment. Results were mostly concordant with those of Fernández-Barral and colleagues [434] and revealed a high number of target genes (including *LRIG*, *SMOC*, *KLF4*, *GRK5*, *RARRES1*, *BCAS*, *TNS4*, and, interestingly, *FGF19*, *MYC*, *FOS*, and *TGFB2*) involved in a wide array of pathways and processes with a large degree of overlap between the two studies and only few discordances. Likewise, no Wnt/ $\beta$ -catenin target genes other than *MYC* were found as regulated by  $1,25(\text{OH})_2\text{D}_3$ . Notably, the canonical VDR-binding motif was the most abundant at peaks in upregulated genes in both the ChIP-seq [434] and the ATAC-seq assays [437]. In the two studies, chromatin VDR-binding sites and accessible sites following  $1,25(\text{OH})_2\text{D}_3$  treatment were located at promoters and in many cases at distant introns and intergenic regions. More recently, Vaughan-Shaw and colleagues [438] have performed a microarray analysis of the effect of the treatment with 50 nM  $1,25(\text{OH})_2\text{D}_3$  during 24 h of organoids of three CRC patients. The authors found 111 differentially expressed genes, many of them involved in the regulation of cell proliferation, differentiation, adhesion, and migration, with a substantial concordance with data from Fernández-Barral and colleagues [434].

At a smaller scale, human intestinal organoids have been used to confirm the action of  $1,25(\text{OH})_2\text{D}_3$  on particular genes. Thus, Bhasin and colleagues reported the upregulation by  $1,25(\text{OH})_2\text{D}_3$  of the uridine phosphatase 1 (*UPP1*) gene, which leads to a reduction of uridine-induced DNA damage in patient-derived CRC organoids [439]. Another study has reported that MDL-811, an allosteric activator of the nicotinamide adenine dinucleotide-dependent deacetylase sirtuin (*SIRT6*), increases histone H3 deacetylation at the *CYP24A1* gene locus. MDL-811 reduces cell proliferation in CRC cell lines and patient-derived organoids and has a synergistic anti-CRC effect in combination with vitamin D in *Apc<sup>min/+</sup>* mice [440]. In addition, the induction by  $1,25(\text{OH})_2\text{D}_3$  of genes involved in the cellular homeostasis of divalent cations (calcium, manganese) previously identified in mice (*TRPV6*, *SLC30A10*, *ATP2B1*) has been confirmed in human duodenal organoids [441].

Mouse organoids have also been used to study vitamin D action on the intestine. Work performed in intestinal organoids from wild-type and mutant *Vdr* mice revealed the regulatory action of vitamin D on the balance between autophagy and apoptosis promoting cell

survival through Atg16l1 and Beclin-1 [325]. Likewise, work in mouse intestinal organoids confirmed the protective effect of vitamin D against injury by restoring the expression of tight junction Zo-1 and claudin 2 proteins that are crucial for intestine barrier function [442]. Contrarily to data in human colon and rectum organoids, a study has reported that  $1,25(\text{OH})_2\text{D}_3$  suppresses cell stemness and proliferation in mouse small intestine organoids, in which it promotes cell differentiation, endoplasmic reticulum stress, and apoptotic death [443], while another study proposed a role of dietary vitamin D in the growth and maturation of mouse intestinal *Lgr5<sup>+</sup>* cells [444]. Curiously, in a mouse model of IBD, locally high  $1,25(\text{OH})_2\text{D}$  concentrations produced by engineered inflammation- and gut-homing macrophages enhanced the migration of intestinal stem cells that later differentiated into mature epithelial cells [445]. It is worth noting that little coincidence exists between target genes of  $1,25(\text{OH})_2\text{D}_3$  in human and mice colon organoids [434], which agrees with global minimal intersection between genes regulated by  $1,25(\text{OH})_2\text{D}_3$  or analogs in the two species found in 94 gene profiling studies [446].

## 5. Animal models

Numerous studies using experimental animals support the protective action of vitamin D,  $1,25(\text{OH})_2\text{D}_3$ , and several  $1,25(\text{OH})_2\text{D}_3$  analogs against CRC.

### 5.1 Diet studies and chemically induced colorectal cancer models

Prolonged feeding (104 weeks) of mice with a “Western-style diet” (WD), containing reduced calcium and vitamin D<sub>3</sub> and the fat level of the average human Western diet, generates single-crypt dysplastic lesions and focal hyperplasia in the colon [447]. In short-term studies (up to 20 weeks) feeding normal rodents with WD induces epithelial cell hyperproliferation in the exocrine pancreas and prostate, and cell hyperplasia and hyperproliferation in the colon and mammary gland. Conversely, WD containing more calcium and vitamin D<sub>3</sub> prevents the hyperproliferation of epithelial cells in those organs [448]. Similar results were obtained with a new Western diet (NWD) that contains additional nutritional risk factors (lower levels of donors to the single carbon pool and lower fiber) and reflects the intake levels of nutrients that are major dietary risk factors for human CRC. The NWD induces colonic tumors when fed to wild-type C57BL/6J mice for two-thirds of their lifespan. Elevating dietary calcium and vitamin D to the upper levels consumed by humans prevents



tumor development. Importantly, tumorigenesis is not altered by a similar increase in folate, choline, methionine, or fiber, each of which is also at low levels in the NWD and is associated with elevated CRC risk [449]. Moreover, C57BL/6J mice fed with the NWD from weaning display higher inflammatory responses than controls, including macrophage-associated crown-like structures in epididymal adipose tissue and elevated serum concentrations of the proinflammatory cytokine IL-1 $\beta$  and its targets, Mcp-1 and Rantes, which were prevented or importantly diminished in the NWD group supplemented with high vitamin D<sub>3</sub> and calcium. Thus, elevating vitamin D and calcium in the NWD can reduce the inflammation associated with increased risk of colon tumor development [450].

The effect of dietary vitamin D on Wnt/ $\beta$ -catenin signaling has been investigated in the healthy colon of mice fed with a modified AIN-93G diet containing either 100 IU vitamin D<sub>3</sub>/kg diet (low vitamin D) or 2500 IU vitamin D/kg diet (high vitamin D). In mice fed with a high vitamin D diet, the levels of Wnt5A and Ror2, which promote degradation of  $\beta$ -catenin, were upregulated, whereas  $\beta$ -catenin and Tcf4 protein expression was reduced. This suggests that vitamin D decreases  $\beta$ -catenin levels in the normal colon partially through this mechanism [220]. Additionally, in the healthy colon of mice, high vitamin D augments the expression of CaSR and differentiation and apoptosis markers, while it reduces that of diverse proliferation markers [341].

Numerous studies have reported that 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces the number of tumors generated by chemical carcinogens in mice or rats (reviewed in Ref. [451]). In a study, female C57BL/6J mice were fed with increasing vitamin D<sub>3</sub> concentrations and treated with AOM followed by the tumor promoter DSS to induce preneoplastic dysplastic lesions. Interestingly, dietary vitamin D<sub>3</sub> concentration correlates inversely with dysplasia score and positively with serum 25(OH)D levels. Therefore, high-dietary vitamin D<sub>3</sub> intake can prevent chemically induced preneoplastic lesions. The maximum impact is achieved in mice that consume more than 2500 IU vitamin D/kg diet [452]. In another study, A/J mice that received the Ro26-2198 analog or vehicle by miniosmotic pump were treated with AOM and DSS, which induces colitis and promotes AOM-induced tumors. Remarkably, Ro26-2198 delays the onset of clinical colitis and blocks the development of the colonic dysplastic foci induced by AOM/DSS treatment. Compared with control animals, AOM/DSS significantly increases Myc, Cox-2, and p-Erk, while Ro26-2198 abolishes these increases [372].

A study examined the potential synergistic chemopreventive effects of vitamin D and metformin against the development of early colon neoplasia in two animal models: 1,2-dimethylhydrazine dihydrochloride

(DMH)-induced CRC rat model and DMH-DSS-induced colitis-associated colon neoplasia mouse model. Combined use of vitamin D and metformin shows a more pronounced effect on reducing the number of colonic aberrant crypt foci and tumors compared with either vitamin D or metformin alone. Likewise, the enhancement of metformin chemopreventive effects by vitamin D is associated with the downregulation of ribosomal S6 protein expression, via the AMPK (IGF-I)/mTOR pathway, while that of vitamin D action by metformin is related to inhibition of the expression of Myc and cyclin D1 proteins via the antagonism of the Wnt/ $\beta$ -catenin pathway [322]. In addition, in AOM-treated Wistar rats, vitamin D and 5-FU synergize and exhibit stronger anticancer effects in combination than in single treatments by modulating the Wnt/ $\beta$ -catenin pathway, TGF- $\beta$ 1 signals, iNOS, Cox-2, and Hsp90 [373].

Benninghoff and colleagues have reported that AOM combined with DSS or *Apc*<sup>Min/+</sup> mouse models of colitis-associated colorectal carcinogenesis (CAC) fed with TWD (a new total WD that emulates a typical WD using available US nutrient intake survey data, which has less calcium and vitamin D than AIN93G diet) developed more severe and more prolonged colitis compared with those fed with AIN93G diet, leading to markedly enhanced colon tumorigenesis. Restoration of calcium and vitamin D inverted the proinflammatory effects of the vitamin D-deficient TWD diet on mucosal gene expression, suggesting a reduction of the symptoms of colitis following DSS treatment, and improved the tumor-promoting effects of TWD [453]. Elimrani and colleagues studied the effect of vitamin D in a mouse model that presented a deficiency in the expression of *Nod2*, a gene that is induced by vitamin D and whose deficiency impairs intestinal epithelial integrity and enhances inflammation. Upon treatment of C57BL/6J and *Nod2*<sup>-/-</sup> mice with AOM and DSS, those with vitamin D deficiency developed more severe colitis than those supplemented with vitamin D, with higher colitis activity index, greater weight loss, increased histological inflammation score, and IL-6 in the mucosa. Overall incidence of colonic tumors was not significantly different between both groups, but vitamin D-deficient mice had higher tumor multiplicity and lower survival rate [454]. After treatment with AOM and DSS, vitamin D-deficient and vitamin D-supplemented mice showed reduced plasma levels of 25(OH)D<sub>3</sub> and reduced expression of *Cyp24a1* and *Vdr* genes (both mice strains) compared with saline-treated controls on the vitamin D-deficient diet. Thus, vitamin D supplementation in both C57BL/6J and *Nod2*<sup>-/-</sup> mice reduced colitis severity and decreased the number of inflammation-associated CRC tumors independently of *Nod2* [454].

## 5.2 Xenografted mice

Human tumor cells implanted subcutaneously into immunosuppressed mice (xenografts) are commonly used as an *in vivo* approach in preclinical anticancer drug development studies. In 1987, Eisman and colleagues reported the suppression by  $1,25(\text{OH})_2\text{D}_3$  of the growth of xenografts generated by VDR-positive human cancer cell lines (COLO206F and COLO239F, established from a CRC and a malignant melanoma, respectively) [455]. Since then, several studies using  $1,25(\text{OH})_2\text{D}_3$  or its analogs have supported these results. In mice implanted with HT29 cells, administration of 0.1- and 0.2- $\mu\text{g}$   $1,25(\text{OH})_2\text{D}_3$  three times/week does not cause significant xenograft growth delay, whereas the synthetic analog Ro25-6760 ( $1,25(\text{OH})_2$ -16-ene-23yne-26,27-hexafluoro-19-nor- $\text{D}_3$  that has increased agonist activity due in part to protection from degradation), at both doses, causes significant tumor growth inhibition in comparison with vehicle-treated mice. Remarkably, in 30% of the mice treated with Ro25-6760, the tumors disappear during treatment and tumor growth does not resume after drug withdrawal. Neither  $1,25(\text{OH})_2\text{D}_3$  nor Ro25-6760 displays a growth-inhibitory effect at any concentration on xenografts generated by VDR-negative SW620 CRC cells [456]. In addition, HT29 xenografts generated in paricalcitol (19-nor- $1,25(\text{OH})_2\text{D}_2$ )-treated mice are smaller and weigh less than those developed in vehicle-treated mice [457]. Accordingly, the calcipotriene-based  $1,25(\text{OH})_2\text{D}_3$  analogs BGP-13 and BGP-15 inhibit the growth of HT29 tumor xenografts [318]. Huang and colleagues have reported that calcitriol suppresses Warburg effect in HT29 cells. It reduces the volume and weight of HT29 xenografted tumors as compared with the control group, and the expression levels of GLUT1 and glycolytic enzymes hexokinase 2 and lactate dehydrogenase A are also lower in the calcitriol group [458]. Moreover, Balb/c mice fed with a vitamin  $\text{D}_3$ -deficient diet (vitamin  $\text{D}_3$ -deficient mice), or a diet supplemented with vitamin  $\text{D}_3$  (vitamin  $\text{D}_3$ -sufficient mice) were injected with MC26 CRC cells. Interestingly, the tumors developed in vitamin  $\text{D}_3$ -sufficient mice are 40% smaller than those generated in vitamin  $\text{D}_3$ -deficient mice. In addition, the RNA expression of *VDR* and *CYP27B1* was higher in vitamin  $\text{D}_3$ -sufficient than in vitamin  $\text{D}_3$ -deficient mice [459].

A study using mice injected with HT29 cells stably overexpressing CYP24A1 or Mock cells and fed with either a high (2500 IU/kg) or a low (100 IU/kg) vitamin  $\text{D}_3$  diet in the presence or absence of soy (20% diet content) showed that, regardless of dietary vitamin  $\text{D}_3$  levels, xenografts overexpressing CYP24A1 grow faster and are heavier and more aggressive than those

generated by Mock cells. Irrespective of vitamin  $\text{D}_3$ , dietary soy increases tumor volume in xenografts that overexpress CYP24A1 [138], which suggests that the combination of vitamin  $\text{D}_3$  and soy could have an antitumorigenic effect only if CYP24A1 levels are normal, or that genistein in soy can inhibit the endogenous and normally regulated CYP24A1 but cannot downregulate forced overexpression of the gene [460].

Padi and colleagues have suggested a role of *miR-627* mediating the suppressive actions of vitamin D on tumor xenografts in mice. Thus, calcitriol induces the expression of *miR-627*, which downregulates Jumonji domain containing 1A (JMJD1A) histone demethylase and suppresses growth of HCT116 cells xenografted tumors in nude mice. Overexpression of *miR-627* prevents growth of these tumors in mice, while blocking the activity of this *miR* inhibits the suppressive effects of vitamin D [461].

*In vivo* models have also been used to investigate the biological effect of combined therapies against CRC. In mice bearing MC38 xenografts, two  $1,25(\text{OH})_2\text{D}_3$  analogs (PRI-2191-tacalcitol and PRI-2205-5,6-trans-isomer of calcipotriol) significantly enhance the antitumor activity of 5-FU, although the results depend on the treatment regimen. In comparison with administration of 5-FU alone, the optimal schedule of combined therapy shows a significant decrease in tumor growth and metastasis and prolongs mouse survival. Both combinations display a synergistic effect and do not cause toxicity. In addition, both analogs prolong the antitumor effect of 5-FU after the completion of drug administration. Potentiation of the activity of capecitabine, a pro-drug of 5-FU, by the  $1,25(\text{OH})_2\text{D}_3$  analogs was, also observed [462]. Furthermore, both combined therapies inhibit the growth of xenografted HT29 cells by a mechanism involving an increase in  $\text{p21}^{\text{CIP}}$  and a decrease in phosphorylated ERK1/2 levels, which may lead to a reduction in thymidylate synthase expression [463]. Moreover, the combined treatment of  $1,25(\text{OH})_2\text{D}_3$  analogs with irinotecan or oxaliplatin significantly inhibits MC38 tumor growth and prolongs mouse survival. Treatment with  $1,25(\text{OH})_2\text{D}_3$  analogs and oxaliplatin also improves the antitumor effects of these compounds in mice bearing HT29 tumors, but an antagonist effect on lifespan prolongation was found [464].

Kim and colleagues have reported that the vitamin D analog calcipotriol can be used as an adjunct to sensitize or prime colorectal cancer cells to oncolytic viro-immunotherapy. Thus, calcipotriol has an additive effect to poxvirus CF33-based viral therapy in an HT29 xenograft model of CRC but not in HCT116 xenografted tumors. RNA-seq and gene expression data analyses demonstrated a downregulation of the JAK/STAT signaling pathway in those HT29 tumors treated with

calcipotriol suggesting that the antiinflammatory properties of vitamin D may enhance the effects of viral therapy in some tumors [465]. Likewise, 1,25(OH)<sub>2</sub>D<sub>3</sub> is also able to sensitize CRC xenograft models to radiotherapy, and proteomic analyses have shown that the mechanism underlying this sensitivity also involves a partial block of the JAK/STAT signaling pathway [466].

### 5.3 Genetic models of colorectal cancer and *Vdr*-deficient mice

#### 5.3.1 Genetic models of CRC

The multiple intestinal neoplasia (Min; *Apc*<sup>Min/+</sup>) model was established from an ethylnitrosourea-treated C57BL/6J male mouse [467] and carries a nonsense mutation at codon 850 of the tumor suppressor *Apc* gene, which causes a truncated *Apc* polypeptide [468] and the consequent accumulation of nuclear β-catenin in the affected cells. This alteration leads to the development of numerous adenomas throughout the intestinal tract of young adult *Apc*<sup>Min/+</sup> mice. The *Apc*<sup>Min/+</sup> animal model is the most widely used for human CRC because of the phenotypic and genetic similarities between murine *Apc*<sup>Min/+</sup> and human FAP, and mutation of the *APC* gene occurs somatically during the development of most sporadic human colorectal tumors. However, the model is not optimal, because mice develop mostly small intestine tumors [468].

Tumor number in female *Apc*<sup>Min/+</sup> mice at 4–5 weeks of age is not affected by 10 weeks of treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> or its analog 1,25(OH)<sub>2</sub>16-ene-19-nor-24-oxo-D<sub>3</sub> (Ro 26-9114). However, a significant decrease in total tumor load (the sum of all polyp areas) over the entire gastrointestinal tract is observed in the analog and the 1,25(OH)<sub>2</sub>D<sub>3</sub> groups [469]. Accordingly, treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub>, QW (QW-1624F2-2-1-α-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26, 27-bishomo-D<sub>3</sub>), or BTW (2,2-dimethylated-19-nor analogue) enhances osteopontin expression, but inhibits that of CD44, the osteopontin receptor implicated in cell growth. These treatments also enhance E-cadherin expression and promote the inhibition of β-catenin nuclear localization and of Tcf1 and Myc protein expression in the *Apc*<sup>Min/+</sup> intestine [470].

Rebel and colleagues demonstrated for the first time an inhibitory effect of moderate UV exposure on the outgrowth and malignant progression of intestinal tumors developed in *Fabp1Cre;Apc*<sup>15lox/+</sup> mice, which at least in part is mediated by vitamin D<sub>3</sub> as UV irradiation increased 25(OH)D<sub>3</sub> serum levels in male and female mice. The tumor load at 7.5 months of age was significantly reduced in both the vitamin D<sub>3</sub>-supplemented and the UV-exposed (daily UV irradiated from 6 weeks of age) groups compared with control animals, but no

reduction in tumor number was found [471]. The effects of a WD were also tested in mice carrying targeted mutations that lead to intestinal and colonic neoplasia. Yang and colleagues generated mice with an insertion of a neomycin phosphotransferase expression cassette at codon 1638 in exon 15 of the *Apc* gene. *Apc*<sup>1638/+</sup> mice develop colonic polyps early in life, and adenomas and carcinomas primarily in the small intestine later in their lifespan. Interestingly, a WD increases the incidence of carcinomas and invasive tumors developed by these mice, which suggests that vitamin D deficiency increases tumor aggressiveness [472].

To avoid issues of species specificity in animal modeling of human disease, Irving and colleagues used two genetic models of familial CRC: the *Apc*<sup>Pirc/+</sup> (*Pirc*) rat and the *Apc*<sup>Min/+</sup> mouse. Treatment with 25(OH)D<sub>3</sub> or two 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs (HP or NC) for 11–16 weeks neither reduced tumor multiplicity nor caused tumor regression of colonic tumors in these models. The authors proposed that the lack of response might be due to reduced tumor *Vdr* expression or to insufficient treatment duration, and it is also possible that the analogs had no effect on animals that were already vitamin D sufficient or that the endoscopic procedure used underestimated the number of tumors [473]. In addition, Irving and colleagues tested whether vitamin D deficiency plays a causative role in tumor development using two strains of the *Apc*<sup>Pirc/+</sup> rat, which contain a truncating mutation in the *Apc* gene and differ in their susceptibility to intestinal tumorigenesis. In the colon, vitamin D deficiency did not increase the development of tumors in either strain and was actually protective in one strain. Unexpectedly, low-dietary calcium combined with vitamin D deficiency significantly suppressed tumor development in the small intestine and colon of both strains [474]. A study using *Apc*<sup>Δ14/+</sup> mice that harbor a frameshift mutation within exon 14 showed that vitamin D<sub>3</sub> does not reduce tumor formation. There was no difference in the number or size of the tumors developed in the small intestine. However, in the colon, there was a trend toward reduction in tumor frequency and load, but this effect did not reach statistical significance. The authors found extensive loss of *Vdr* expression in these tumors, which could explain the lack of CRC protection afforded by vitamin D<sub>3</sub> in this model [475]. Similarly, a study on hyperplastic colonic lesions of *CAC;Apc*<sup>Δ580/Δ580</sup> mice (transgenic mice with colon-specific deletion of both *Apc* alleles) has shown that short-term exposure to pharmacological doses of 1,25(OH)<sub>2</sub>D<sub>3</sub> does not suppress colonic Wnt/β-catenin signaling. Although 1,25(OH)<sub>2</sub>D<sub>3</sub> activates classical *Vdr* target genes (e.g., *Cyp24a1* and *Trpv6*) in the colon of *CAC;Apc*<sup>Δ580/Δ580</sup> and control mice, it does not reduce the elevated RNA levels of β-catenin target genes or the high proliferation index observed in *CAC;Apc*<sup>Δ580/Δ580</sup>



mice. Again, these results could be explained by the lower levels of *Vdr* RNA and protein in the colon of *CAC;Apc<sup>4580/4580</sup>* mice than in that of control animals, which indicates a loss of colon responsiveness to vitamin D compounds. It also seems that the use of 1,25(OH)<sub>2</sub>D<sub>3</sub> or analogs to suppress  $\beta$ -catenin signaling is limited and that tumors with complete loss of *Apc* function may be resistant to vitamin D action [476].

In another study, upon AOM + DSS treatment, the levels of *Il-6* and *Tnf- $\alpha$*  RNA were higher in untreated mice than in those supplemented with vitamin D. Accordingly, while the untreated group showed an inflammatory cell infiltration and interstitial connective tissue hyperplasia, mice treated with 1500 IU or 3000 IU of vitamin D had a relatively clear structure of colon layers with only mild edema and less inflammation. Vitamin D induced mucin (*Muc*)2 expression, which can be considered as a beneficial response that could enhance the protective action of the mucus barrier, but inhibited *Muc1* gene expression, the major membrane-bound mucin in the colon that plays a role in cell signaling and damage repair [477]. In this study, vitamin D diet modulated the expression level of key genes in microbial defense and injury-repair responses. Thus, it upregulated resistin-like molecule  $\beta$ , a goblet cell secretory hormone that enhances colon barrier function and regulates colitis, and there was a trend toward downregulation of regenerating islet-derived protein 3  $\gamma$ , which is an antibacterial C-type lectin [477].

Interestingly, *Smad3*<sup>-/-</sup> mice (which have defective Tgf- $\beta$ -signaling and develop colitis and colitis-associated CRC after the administration of DSS) treated with DSS and fed with a vitamin D-null diet showed improved survival, reduced incidence and severity of colonic dysplasia, and decreased colon tumor incidence compared with mice fed a control diet. These effects correlated with increased epithelial cell proliferation and repair at early stages of the disease. As suggested by the authors, vitamin D deficiency may be beneficial in some cases of CAC, possibly by promoting intestinal healing [478].

### 5.3.2 *Vdr*-deficient mice

Four groups have independently generated *Vdr*-deficient mice (*Vdr*<sup>-/-</sup>) by disrupting of the *Vdr* DNA-binding domain using different targeting constructs and mouse strains. Abnormalities in all *Vdr*<sup>-/-</sup> mice develop only after weaning, showing a phenotype with the appearance of hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, alopecia, and severely impaired bone formation, leading to growth retardation, severe rickets, and osteomalacia; the typical features of vitamin D-dependent rickets type II [479–482]. The expression of 8-hydroxy-20-deoxyguanosine (8-OHdG), a marker of oxidative

DNA damage, is significantly augmented in the colon of *Vdr*<sup>-/-</sup> mice. In addition, an increased level of the proliferation marker PCNA is observed in *Vdr*<sup>-/-</sup> mice colon progeny. The presence of only a partially functional 1,25(OH)<sub>2</sub>D<sub>3</sub>/*Vdr* system in the colon of *Vdr*<sup>+/-</sup> animals can reduce by 40% the expression of 8-OHdG compared with *Vdr*<sup>-/-</sup> animals. Thus, the partial or complete lack of 1,25(OH)<sub>2</sub>D<sub>3</sub>/*Vdr* action results in colonic premalignant alterations [122].

Two independent groups generated *Apc*<sup>Min/+</sup>*Vdr*<sup>-/-</sup> mice by breeding. No differences were reported in the number of small intestinal and colonic tumors between *Apc*<sup>Min/+</sup>*Vdr*<sup>+/-</sup> and *Apc*<sup>Min/+</sup>*Vdr*<sup>-/-</sup>. However, size of the tumors was significantly higher in *Apc*<sup>Min/+</sup>*Vdr*<sup>-/-</sup> mice [247,483]. These observations suggest that the increased tumor burden in *Apc*<sup>Min/+</sup>*Vdr*<sup>-/-</sup> mice is probably due to loss of the inhibitory effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on tumor growth rather than on tumor initiation in the intestine [483]. Moreover, both aberrant crypt foci and carcinomas in *Apc*<sup>Min/+</sup>*Vdr*<sup>-/-</sup> mice showed higher expression of nuclear  $\beta$ -catenin and  $\beta$ -catenin/TCF target genes than those developed in *Apc*<sup>Min/+</sup>*Vdr*<sup>+/-</sup> mice. Consistently, *Vdr* depletion in cultured CRC cells increases  $\beta$ -catenin nuclear levels and transcriptional activity and abrogates the capacity of 1,25(OH)<sub>2</sub>D<sub>3</sub> to promote the relocation of  $\beta$ -catenin from the nucleus to the plasma membrane and to inhibit  $\beta$ -catenin/TCF target genes [247]. Moreover, total *Vdr* knockout (*Vdr*<sup>-/-</sup>) and intestinal epithelial *Vdr* knockout (*Vdr*<sup>ΔE1C</sup>) mouse models led to lower expression of claudin-5 in colon tissue, while overexpression of *Vdr* in intestinal epithelial cells led to an increase of claudin-5 and a decrease of the inflammatory cytokines *Il-1 $\beta$*  and *Il-17* compared with control mice [484].

The summary from studies using animal models and diverse experimental approaches appears to be that vitamin D compounds protect against intestinal carcinogenesis provided that VDR is expressed in CRC cells and that lack of VDR per se does not cause CRC, but it increases CRC susceptibility/risk in response to insults, mutations, or an unhealthy diet.

## 6. Conclusion

The data presented show that 1,25(OH)<sub>2</sub>D<sub>3</sub> exerts a wide array of effects on several types of cells that collectively support a protective action against CRC (Fig. 89.3). 1,25(OH)<sub>2</sub>D<sub>3</sub> affects not only colorectal carcinoma cells but also nontumoral CAFs and possibly other stromal cell types, as well as colorectal organoids composed of normal or cancer stem cells and their progeny. Accordingly, vitamin D compounds inhibit the tumorigenic process in a variety of in vivo animal models of CRC. Given the key importance of the Wnt/



$\beta$ -catenin signaling pathway in CRC, its multilevel antagonism in carcinoma cells appears as an important mechanism of  $1,25(\text{OH})_2\text{D}_3$  action. Therefore, the results of preclinical studies agree with a large number of epidemiological studies showing CRC as the neoplasia most strongly associated with vitamin D deficiency and are highly indicative of causality. However, this contrasts with the unclear results of intervention/supplementation studies, which may be due to deficiencies in their design and analysis, and to our insufficient knowledge of the optimal vitamin D compounds dosage and administration. The extreme health impact of CRC warrants the continuation of the study of vitamin D action.

## 7. Summary points

- $1,25(\text{OH})_2\text{D}_3$  inhibits the proliferation and invasiveness and promotes the differentiation of colon carcinoma cells.
- $1,25(\text{OH})_2\text{D}_3$  antagonizes the Wnt/ $\beta$ -catenin pathway in colon carcinoma cells by a variety of mechanisms.
- $1,25(\text{OH})_2\text{D}_3$  reduces cell proliferation and protumoral phenotype in cultures of CRC patient-derived stromal tumor fibroblasts and in 3D colon organoids.
- $1,25(\text{OH})_2\text{D}_3$  attenuates tumor growth in several animal models of CRC.

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# Vitamin D in the management of lung cancer: influence of tumor stage, tumor genotype, and treatment modality

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## OBJECTIVES

- Lung cancer has always been viewed and studied as a heterogenous disease. This chapter seeks to explore the role of vitamin D in lung cancer risk and survival in the context of disease heterogeneity.
- Our goal is to promote a thoughtful precision guided future evaluation of vitamin D in lung cancer. This chapter offers an insight into the current knowledge of subtype-specific antitumor activity of vitamin D to facilitate such an evaluation.

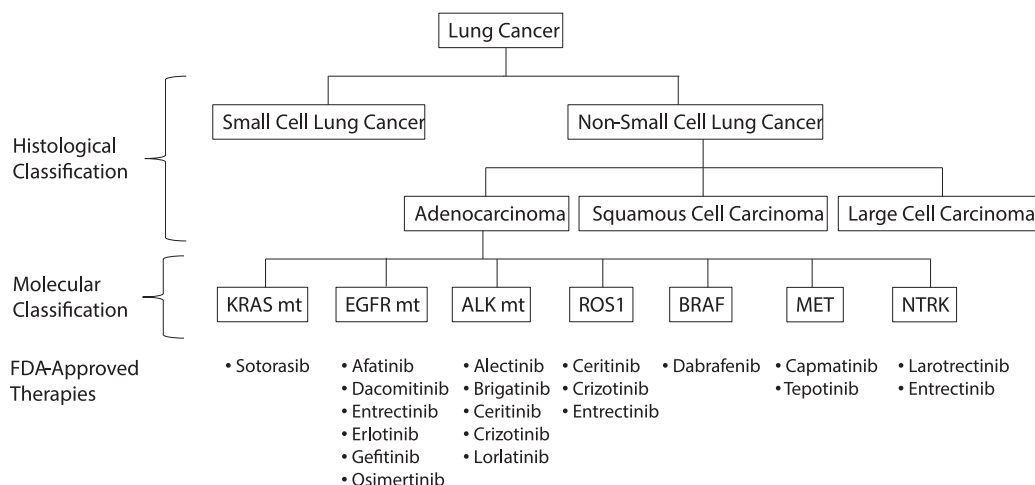
chemoprevention in individuals at high risk for disease, improved adjuvant therapies for early-stage disease, and personalized therapies for advanced disease. Is there a role for vitamin D in this context? A PubMed search of “vitamin D and lung cancer” resulted in a list of nearly 500 publications. Published results do not support a ubiquitous role for vitamin D in preventing progression of lung cancer. Rather, benefit of vitamin D supplementation may be restricted to specific patient subsets. This chapter will consider which patients have potential to respond to vitamin D and why such patient-specific differences exist. Ultimately, well-informed clinical trials of precision vitamin D supplementation will be needed to test hypotheses and establish efficacy in the management of lung cancer.

Central to the theme of this chapter is recognition that lung cancer is not a single disease. Rather, it is a collection of diseases that are distinguished based on their histology and molecular profiles (Fig. 90.1). Historically, lung cancer is categorized based on histology (small-cell lung cancer [SCLC] versus non-small cell lung cancer [NSCLC]). SCLC encompasses 10%–15% of all lung cancers. Almost all patients diagnosed with SCLC have a history of smoking. As the name suggests, these tumors are comprised of cancer cells that are small and round in appearance. SCLCs grow and spread faster than NSCLCs, and patients diagnosed with SCLC have a dismal 5-year survival rate of only 6% [5]. One-third of patients will be diagnosed with limited stage SCLC,

## 1. Introduction

Lung cancer is the second most frequently diagnosed cancer in men and women, with >236,000 cases expected to be diagnosed in 2022 [1]. Lung cancer remains the leading cause of cancer-related deaths in both sexes, despite advances in diagnostics, surgery, and therapy. Tobacco smoking is a primary cause of disease, accounting for nearly 85% of all lung cancers [2]. Strategies to reduce lung cancer mortality are multifold and include enhanced lung cancer screening, tobacco cessation,





**FIGURE 90.1 Classification and treatment of lung cancer.** Lung cancer is subclassified based on histology and molecularly, based on the presence of oncogenic driver mutations. Oncogenic drivers in LSCC (reviewed in Ref. [3]) are distinct from the validated oncogenic drivers found in LUAD. To date, molecularly targeted therapies have had a greater impact in LUAD compared with LSCC. In the United States, an oncogenic driver is identified in 64% of LUAD [4]. *EGFR* mutations (mt) and *ALK* translocations are enriched among females, younger patients, and lifetime never smokers. *KRAS* mt are more frequently observed in patients with a significant history of tobacco smoke exposure. FDA-approved drugs that target specific oncogenic drivers in LUAD are shown.

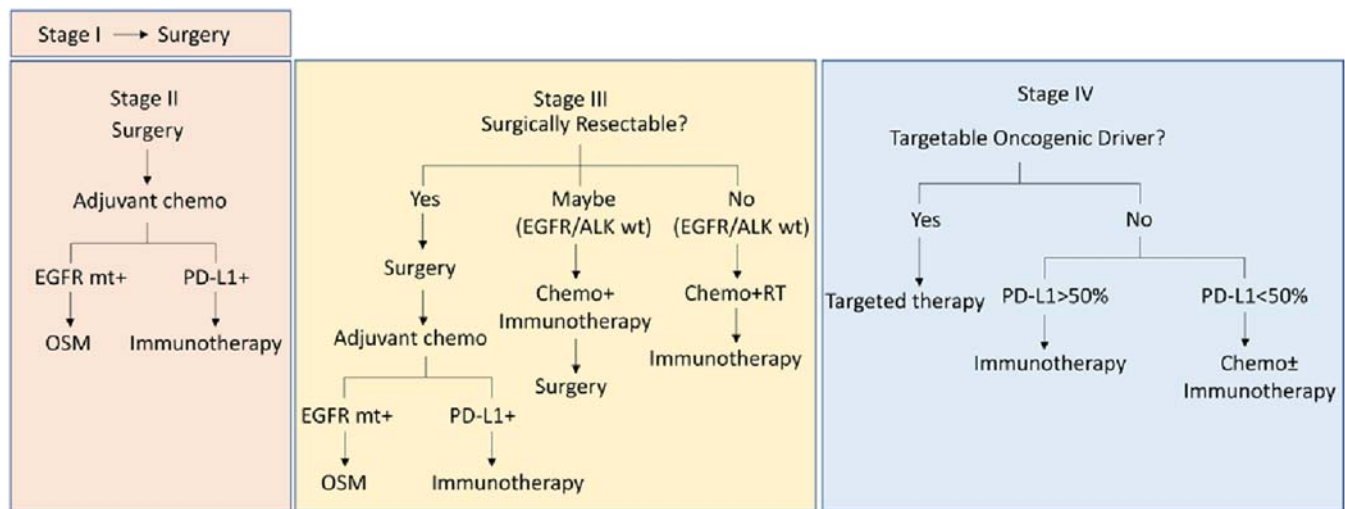
where more opportunity for therapeutic intervention exists [6]. Treatment options for limited stage SCLC include surgery, chemotherapy, radiation, a combination of chemotherapy and radiation (chemoradiation), and immunotherapy [7]. Most cases are treated in a multi-therapy approach, according to the National Comprehensive Cancer Network (NCCN) guidelines. Treatment of limited-stage SCLC produces 5-year survival rates of up to 27%. Two-thirds of SCLC patients will present with extensive disease and have poor prognosis. Patients with extensive SCLC receive systemic therapy that typically includes multiple cycles of chemotherapy with a platinum-based doublet. Chemotherapy may be combined with contemporary immunotherapy or radiation therapy. Radiation therapy becomes a more common treatment method when SCLC has spread to the brain. Overall survival for extensive stage SCLC is approximately 10 months, with most patients developing recurrent disease after initial treatment [7]. Very few studies have investigated the role of vitamin D specifically in SCLC. Therefore, this disease will not be considered further in the chapter.

NSCLC accounts for 80%–85% of all lung cancers. There are three major subtypes of NSCLC, which differ in their cell of origin, anatomic location, and treatment options (Fig. 90.1). Adenocarcinoma of the lung (LUAD) represents around 40% of all NSCLC cases and arises from secretory cells in the distal airways. Squamous cell carcinoma of the lung (LSCC) accounts for 30% of NSCLC cases and arises from bronchial epithelial cells that line the central airways. LSCC is more tightly associated with smoking than LUAD. Large

cell carcinoma is the least common of the three major subtypes, accounting for 5%–10% of all cases, and can appear in any part of the lung. These cancers grow and spread quickly and can have similar features to those of SCLC histology.

Treatment options for NSCLC are dictated by numerous factors including histologic subtype, staging, and in the case of LUAD, by tumor mutation profiles and expression of the immune checkpoint, PD-L1 (Fig. 90.2, [8]). The TNM system is used for NSCLC staging. T denotes tumor size. N relates to the number of lymph nodes to which the cancer has spread. M stands for metastasis and indicates that cancer has spread across or out from the lung to another part of the body. A lower TNM score (stage I) carries a better prognosis than a higher score (stage IV). Patients diagnosed with early-stage NSCLC receive surgery with curative intent. Surgery may also be used in patients with stage III disease, but presurgical (neoadjuvant) or postsurgical therapies are also required. Patients diagnosed with stage IV cancers receive systemic therapy. Remarkable gains in survival of patients with advanced NSCLC have been achieved over the past decade due to the incorporation of targeted therapies and immunotherapies (immune checkpoint inhibitors) into treatment regimens. Success of these therapies in advanced disease has led to their subsequent evaluation and approval by the FDA for use at earlier stages. For example, osimertinib (OSM) received approval for use in advanced disease in 2015 and as adjuvant therapy in early-stage disease in late 2020.

Targeted therapy relies on the identification of molecular drivers of the cancer. Oncogenic drivers encode



**FIGURE 90.2** Lung cancer treatment varies by stage at diagnosis and molecular features of tumor. Algorithms that are used to identify stage-specific and genotype-specific treatments for NSCLC are shown. Illustration is meant to be representative and is not inclusive for all possible therapies that may be used. Surgery is performed with curative intent for stage I and stage II disease. Targeted therapies and immunotherapies, which have revolutionized the treatment of advanced disease, are now safely and effectively deployed at earlier disease stages.

proteins whose activity is required for tumor cell survival: Inhibition of the abnormal protein results in tumor growth suppression [9–11]. Multiple FDA-approved drugs have been developed that selectively and effectively inhibit several of the oncogenic drivers of LUAD including the epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK), and BRAF (v-Raf murine sarcoma viral oncogene homolog B) (Fig. 90.1). In May 2021, the first drug to selectively inhibit KRAS G12C (sotorasib) was FDA-approved [12]. A 1-year benefit in overall survival is obtained in patients diagnosed with advanced LUAD who receive therapy that is matched to the oncogenic driver expressed in their lung tumor (so-called, personalized medicine) versus those who do not receive genotype-matched therapy [4]. As a result, it is standard practice to sequence lung tumors from advanced NSCLC patients prior to therapy selection. Although these targeted therapies provide survival benefit of 1–2 years in some cases, drug resistance will inevitably develop [13–15]. This highlights the need to develop innovative approaches to extend the durability and efficacy of targeted therapies.

Immunotherapy involves the administration of antibodies (referred to as immune checkpoint inhibitors) that block the interaction between an immune checkpoint protein expressed on CD8+ T cells (for example, PD-1) and its cognate partner expressed on lung tumor cells (PD-L1). The binding of PD-L1 to PD-1 inhibits T cell killing of the tumor cell. By disrupting this inhibitory interaction, immune checkpoint inhibitors promote T cell-mediated antitumor activity. Immune checkpoint inhibitors that are approved for use in NSCLC include

nivolumab (anti-PD-1), pembrolizumab (anti-PD-1), atezolizumab (anti-PD-L1), durvalumab (anti-PD-L1), and ipilimumab (anti-CTLA4). The objective response rate to immune checkpoint inhibitors is approximately 30%. Biomarkers that distinguish responders versus nonresponders have been developed to avoid use of an ineffective therapy that can have serious immune-related toxicities. Expression of the biomarker PD-L1, tumor mutation burden, and microsatellite instability are now routinely used to identify patients who are more likely to derive benefit from immunotherapy [16].

## 2. Vitamin D may have tumor cell intrinsic and tumor cell extrinsic activities in lung cancer

The presence of vitamin D receptor (VDR) in cells/target tissues is required for generating a response to vitamin D metabolites. The VDR is expressed in premalignant lesions and some lung tumors [17], raising the possibility that tumor-intrinsic vitamin D signaling occurs and may be exploited in the chemoprevention or treatment of lung cancer. However, VDR is also expressed in a variety of immune cells, including T cells, where it regulates differentiation and effector function [18–20]. Vitamin D signaling within immune cells may be critically important in modulating the efficacy and toxicity of immune checkpoint inhibitors. The existence of VDR in multiple cell types within the tumor microenvironment and the differential importance of vitamin D signaling events in different disease and treatment contexts prevents a one-size-fits all response to the question of whether vitamin D plays a role in lung

cancer. To address this complexity, we summarize current knowledge regarding the role of vitamin D across the lung cancer treatment continuum based on disease stage and treatment type.

### 3. The role of vitamin D in the chemoprevention of non–small-cell lung cancer

The chemopreventive effects of vitamin D metabolites have been tested using both murine models of LUAD [21] and LSCC [22]. LUAD is induced in A/J mice using the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [23]. The ability of 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) to modify lung tumorigenesis in the NNK model was investigated by Mernitz et al. [21]. Mice received control, AIN-93M diets, or the same AIN-93M diet supplemented with 1,25(OH)<sub>2</sub>D<sub>3</sub> (2.5 or 5.0 µg/kg diet) for 3 weeks before and 17 weeks after NNK administration. 1,25(OH)<sub>2</sub>D<sub>3</sub> supplementation resulted in statistically significant and dose-dependent decreases in both lung tumor incidence (reduced 82% at 5.0 µg/kg 1,25(OH)<sub>2</sub>D<sub>3</sub>) and multiplicity (reduced 98% at 5.0 µg/kg 1,25(OH)<sub>2</sub>D<sub>3</sub>). However, 1,25(OH)<sub>2</sub>D<sub>3</sub> supplementation at this dose was not well tolerated: Mice experienced significant weight loss and dose-dependent kidney calcification, consistent with toxicity due to hypercalcemia.

LSCC develops from the normal bronchial epithelium through a progressive series of molecular and histologic changes (reviewed in Ref. [24]). Histologic progression includes basal cell hyperplasia, squamous metaplasia, dysplasia, carcinoma in situ, and invasive LSCC. The VDR is expressed within histologically normal bronchial epithelium, metaplastic lesions, and dysplastic lesions [17]. It is therefore plausible that VDR signaling can be activated within premalignant lesions by dietary vitamin D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> to delay development of LSCC. To study the chemopreventive effects of vitamin D in this disease context, Mazzilli et al. [22] employed the N-nitroso-tris-chloroethylurea (NTCU) model. NTCU is a chemical carcinogen that induces premalignant bronchial lesions that progress to frank lung SCC in mice [25]. SWR/J mice were maintained on a deficient diet containing no vitamin D<sub>3</sub> or a sufficient diet containing 2000 IU/kg vitamin D<sub>3</sub>. Mice on either diet received NTCU on a chronic basis. At study termination, the percentage of the mucosal surface of large airways occupied by high-grade dysplastic lesions was 22.7% in vitamin D–deficient mice and 8.7% in vitamin D–replete mice. Proliferation and inflammation were both significantly increased in NTCU-treated, vitamin D–deficient mice compared to NTCU-treated, vitamin D–replete mice. These studies support the conclusion that vitamin D repletion reduces the progression of

premalignant lesions and so may suppress development of lung SCC. Interestingly, Mazzilli et al. also evaluated the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> supplementation (80 µg/kg weekly, ip) on disease progression in the NTCU model. 1,25(OH)<sub>2</sub>D<sub>3</sub> decreased the percentage of high-grade dysplasia in vitamin D–deficient mice but had little to no effect in vitamin D–replete mice. No hypercalcemic toxicity was observed with this weekly dosing regimen. These data suggest that 1,25(OH)<sub>2</sub>D<sub>3</sub> supplementation among individuals with documented vitamin D deficiency may reduce premalignant changes in the bronchial epithelium. However, there was little benefit detected from 1,25(OH)<sub>2</sub>D<sub>3</sub> supplementation in vitamin D–replete mice.

If vitamin D plays a role in the prevention of lung cancer, one would predict that an inverse association exists between vitamin D exposure and risk of developing lung cancer. Indeed, multiple ecologic and epidemiologic studies have been conducted to ascertain this relationship. Variable associations have been found (for example, see Refs. [26–28]). However, two recent meta-analyses provide consistent evidence of protective effects of vitamin D. Zhang et al. tested the association between vitamin D and lung cancer risk using data from nine prospective cohort studies and three nested case–control studies [29]. They determined that the relative risk of lung cancer was 0.84 for high versus low vitamin D ( $P < .001$ ). However, serum 25(OH)D<sub>3</sub> concentrations were not reported, so the level of 25(OH)D<sub>3</sub> required for protection was not established. Chen et al. provided insight into this issue by performing a dose–response metaanalysis using data pooled from 10 prospective studies [30]. Their analysis identified a statistically significant 5% decrease in lung cancer risk for each 10 nmol/L increase in 25(OH)D<sub>3</sub>. The greatest reduction in lung cancer risk was detected at serum 25(OH)D<sub>3</sub> levels between 53 and 90 nmol/L. However, 25(OH)D<sub>3</sub> levels were measured at different laboratories and by different methods, which could have influenced the results. To overcome this limitation, Muller et al. examined the relationship between prediagnostic 25(OH)D<sub>3</sub> concentration (determined by gold standard LC-MS/MS assay in a single, DEQAS-certified laboratory) and lung cancer risk using >5300 cases and carefully matched controls selected from the Lung Cancer Cohort Consortium [31]. Participants were from Asia, Australia, Europe, and the United States, and 76% of the cohort was comprised of current- or former-smokers. No evidence was obtained for risk reduction by vitamin D.

Ultimately, randomized clinical trials will be required to definitively establish vitamin D effects on lung cancer risk. In this regard, multiple randomized, placebo-controlled trials of vitamin D<sub>3</sub> supplementation to prevent cancer have been recently completed including the Finnish Vitamin D Trial [32], the VITAL study [33],

**TABLE 90.1** The effect of vitamin D supplementation on cancer room.

Study	Baseline 25(OH)D <sub>3</sub> (ng/mL) <sup>a</sup>	Intervention	Average increase 25(OH)D <sub>3</sub> (ng/mL) <sup>a</sup>	HR vitamin D <sub>3</sub> versus placebo
Finnish trial	73.7	1,600 IU vitD <sub>3</sub> /day for 5 years	+9.4	1.14 (95% CI, 0.75–1.72; <i>P</i> = .55)
		3,200 IU vitD <sub>3</sub> /day for 5 years	+17.4	0.95 (95% CI, 0.61–1.47; <i>P</i> = .81)
Vital	77	2,000 IU vitD <sub>3</sub> /day for 5 years	+22	0.96 (95% CI, 0.88–1.06; <i>P</i> = .47)
ViDA	66.2	200,000 IU bolus then 100,000 IU monthly for median 3.3 y	+20	1.01 (95% CI, 0.81–1.25; <i>P</i> = .95)

<sup>a</sup>Among participants who had blood samples available for analysis.  
HR, hazard ratio; IU, international units.

and the ViDA study [34]. All three studies focused on older adults. Participants were enrolled regardless of baseline vitamin D status. Summary details for each trial are provided in Table 90.1. Supplementation was consistently effective at increasing serum 25(OH)D<sub>3</sub> but did not reduce cancer incidence in the overall study populations (see Hazard Ratios, Table 90.1). However, protective effects were observed in a specific subset of VITAL participants: Vitamin D<sub>3</sub> supplementation was associated with a significant decrease in total cancers but only among individuals with normal body mass index [33]. This observation supports the idea that demographic factors such as obesity modify response to vitamin D<sub>3</sub> supplementation. No information about lung cancer incidence in placebo versus supplementation arms was reported in any of the trials. Thus, the impact of intervention specifically with regard to lung cancer risk remains undefined.

#### 4. The role of vitamin D in management of early-stage lung cancer

In addition to the potential role of vitamin D in slowing the development of lung cancer described above, several epidemiologic studies implicate vitamin D in controlling growth of established lung cancers. The first evidence that vitamin D status may play a role in established disease came in 2005, when an association between season of lung cancer resection as well as vitamin D intake and outcomes in early-stage NSCLC was reported [35]. Patients who underwent surgery

during summer months and had high vitamin D intake had improved overall and recurrence-free survival (RFS) compared with patients who had surgery during the winter with the lowest vitamin D intake [35]. Zhou et al. subsequently measured circulating 25(OH)D<sub>3</sub> in relation to outcomes using the same cohort of patients diagnosed with early-stage NSCLC [36]. More than 50% of subjects were classified as vitamin D<sub>3</sub>-deficient. Among patients with stage IB-IIIB NSCLC, those with the highest 25(OH)D<sub>3</sub> levels ( $\geq 21.6$  ng/mL) had significantly better RFS and overall survival (OS) than those with the lowest 25(OH)D<sub>3</sub> levels ( $< 10.2$  ng/mL).

Quite excitingly, protective effects of vitamin D<sub>3</sub> supplementation among patients with early-stage NSCLC have now also been observed in a prospective, randomized, placebo-controlled trial [37]. Patients who underwent surgery for NSCLC were randomized to receive placebo (*n* = 78) or 1200 IU vitamin D<sub>3</sub>/d (*n* = 77) for 1 year. The vitamin D<sub>3</sub> dose selected based on prior studies by group in Parkinson's disease. Patients (the majority of which had LUAD) received appropriate chemotherapy based on their diagnosis. Supplementation was effective. The average serum 25(OH)D<sub>3</sub> concentration in the supplemented group went from 21 to 39 ng/mL, whereas little change was observed in the group that received placebo. In the overall study cohort, no effect of supplementation on either RFS or OS was found. However, a significant effect was found within the subset of patients who had LUAD and were vitamin D deficient ( $< 20$  ng/mL) at baseline. RFS was 50% for placebo and 86% for the supplemented group (*P* = .04). OS was 48% for placebo and 91% for supplemented group (*P* = .02). A major conclusion from this trial was that vitamin D<sub>3</sub> supplementation is not likely to improve outcomes for all patients. Rather, specific patients (those with LUAD who are vitamin D deficient) will derive benefit. It is important to note that this trial was conducted in Japan, where *EGFR* mutations represent the predominate oncogenic driver of LUAD (44% of cases harbor activating mutations in *EGFR*). Results may portend a better effect of vitamin D specifically in *EGFR* mutant NSCLC, although this remains to be determined.

#### 5. The role of vitamin D in the management of advanced lung cancer

Owing to major discoveries in the field of lung cancer biology, advanced lung cancer standard of care has undergone a dramatic transformation in the recent decade. For many patients, targeted therapies and immune checkpoint inhibitors have replaced traditional cytotoxic chemotherapeutics. In this section, we will



provide some insights into the evolving role of vitamin D in the setting of this novel treatment paradigm.

### 5.1 *EGFR* mutations (mt) mark a subset of advanced LUAD that are vitamin D responsive

Although vitamin D signaling capacity and its potential antitumor activity in the lung may be dictated by tumor mutation profile, investigations into the role of vitamin D in lung cancer that were conducted before the advances of personalized medicine generally did not take into consideration tumor-specific genetic alterations. As genotype-matched therapies are increasingly used in contemporary management of advanced lung cancer, prognostic value of vitamin D status in defined molecular subtypes of lung cancer has come into the spotlight.

Several studies investigated VDR expression and antiproliferative effects of  $1,25(\text{OH})_2\text{D}_3$  in distinct molecular subclasses of NSCLC [38–40]. Cell lines were selected, which harbored mutually exclusive activating mutations in either the *KRAS* (A549, 128-88T) or *EGFR* genes (HCC827, H1975). Activating *EGFR* mutations are more frequently observed in females and non-smokers, whereas oncogenic *KRAS* mutations are more frequent in males and are associated with smoking [41]. Intriguingly, *KRAS* mutant lines expressed VDR at lower levels than the *EGFR* mutant cell lines. *EGFR* mutant cell lines displayed greater induction of VDR target genes and greater susceptibility to  $1,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition than *KRAS* mutant cell lines. Similarly, PRI-2191, a potent VDR agonist, exerted higher antiproliferative activity and cell cycle arrest in *EGFR* mutant versus *KRAS* mutant lung cancer cells [42].

To determine whether *EGFR* mutant NSCLC cells were vulnerable to vitamin D-mediated growth inhibition in vivo, Verone-Boyle et al. fed mice bearing HCC827 subcutaneous xenografts research diets that varied in vitamin  $\text{D}_3$  content (100 IU–10,000 IU vitamin  $\text{D}_3$ /kg diet) [43]. Tumor growth (volume distribution and growth rate) was reduced significantly in mice that were fed the high dose 10,000 IU vitamin  $\text{D}_3$ /kg diet versus the low dose 100 IU vitamin  $\text{D}_3$ /kg diet. Verone-Boyle further demonstrated that VDR protein is expressed at detectable levels in 18/18 primary human lung tumors that harbor activating mutations in the *EGFR* gene [43]. A recent report demonstrated that circulating levels of  $25(\text{OH})\text{D}_3$  of  $>30$  ng/mL were predictive of a longer progression free survival in advanced NSCLC patients whose tumors harbored activating mutations in the *EGFR* gene (and received erlotinib), but not in those whose tumors were *KRAS* mutant (and received

cytotoxic chemotherapy) [44]. Oncogenic *KRAS* alterations are reported to be at least twice as frequent as *EGFR* mutations in lung cancer patients within the United States [45,46]. Consequently, prognostic value of vitamin D status may not be evident in molecularly nonselected cohorts, where a large fraction of patients is likely to have vitamin D–refractory *KRAS*-mutant tumors.

It is not yet known why *EGFR* and *KRAS* mutant NSCLC cells differ in VDR expression and  $1,25(\text{OH})_2\text{D}_3$  sensitivity. Zhang et al. hypothesized that mutation-related differences occur because *KRAS* and *EGFR* somatic alterations are differentially associated with smoking [38]. It is known that cigarette smoke exposure stimulates DNA hypermethylation and gene silencing [47–49] and that the VDR locus is subject to epigenetic control [50]. In light of this information, it seems possible that prior smoke exposure resulted in hypermethylation and long-term silencing of the VDR promoter specifically in *KRAS* mutant NSCLC cells. One could further predict that NSCLC cell lines derived from never-smokers and light smokers are more likely to express VDR and respond to  $1,25(\text{OH})_2\text{D}_3$  than NSCLC cell lines derived from smokers. This appears to be the case: H2228 (donor was nonsmoker), H3122 (harbors ELM4-*ALK* translocation associated with never or light smoking status), HCC827 (harbors *EGFR* mutation associated with never or light smoking status), and H2347 (donor was nonsmoker) cells were all tested for vitamin D sensitivity as part of larger NSCLC cell line panels [40,51,52]. In each study, the “nonsmoker” cell lines rank among the most sensitive to vitamin D–mediated growth inhibition. Cumulatively, these data imply that never or light smoking status, *EGFR* gene mutations, or *ALK* translocations may be used to identify those NSCLC patients who are likely to derive benefit from vitamin D supplementation.

### 5.2 24-Hydroxylase, encoded by the *CYP24A1* gene, has adverse effects in LUAD

In addition to high levels of VDR, lung tumor models that are intrinsically responsive to vitamin D express low levels of *CYP24A1*. *CYP24A1* encodes 24-hydroxylase, the primary enzyme responsible for catabolic inactivation of  $1,25(\text{OH})_2\text{D}_3$  [53]. *CYP24A1* expression is increased by  $1,25(\text{OH})_2\text{D}_3$  in normal target tissues including kidney, colon, intestine, and bone [54–56]. Inactivation of either VDR or *CYP24A1* (via inherited mutations or molecular approaches) results in elevated blood levels of  $1,25(\text{OH})_2\text{D}_3$ , indicating that VDR-dependent activation of *CYP24A1* is required for efficient  $1,25(\text{OH})_2\text{D}_3$  catabolism by normal tissues [57–60]. Physiologically, *CYP24A1* induction by

1,25(OH)<sub>2</sub>D<sub>3</sub> attenuates its own actions and prevents accumulation of toxic doses of the hormone.

*CYP24A1* is also expressed in lung cancer, where it promotes 1,25(OH)<sub>2</sub>D<sub>3</sub> catabolism and loss of antitumor activity. The expression of *CYP24A1* in lung cancer cells was first described in 1999 [61]. Shortly thereafter, it was demonstrated that *CYP24A1* mRNA and 24-hydroxylase are overexpressed in primary human lung tumors [62–64]. Shiratsuchi and colleagues reported a direct correlation between *CYP24A1* levels and tumor cell proliferation rates in vitro and in vivo [65]. In patients diagnosed with early-stage LUAD, *CYP24A1* mRNA expression is independently prognostic of survival: 5-year survival rates are 81% in individuals whose tumors express low levels of *CYP24A1* and 42% for those with high *CYP24A1* expression [64]. Studies conducted in preclinical models of lung cancer revealed that the oncogenic potential of *CYP24A1* is not only due to its function as a vitamin D degrader but also linked to its noncatalytic activity [66]. *CYP24A1* was shown to promote cell cycle progression by direct inhibition of APC, a ubiquitin ligase, critical for control of cell cycle.

It is generally agreed on that *KRAS*-mutant tumors, including those originating in lung, are not only deficient in VDR, but also express high levels of *CYP24A1* [38,43,67]. The VDR<sup>low</sup> *CYP24A1*<sup>high</sup> phenotype of *KRAS*-mutant lung tumors may be responsible for their apparent lack of response to vitamin D.

### 5.3 Combining vitamin D with targeted therapeutics

A growing body of evidence suggests that future investigations of vitamin D in the context of advanced lung cancer may be best focused on the most responsive subtypes and in combination with other therapeutic agents. *EGFR*-mutant NSCLC is emerging as a primary candidate for evaluation of vitamin D–based combination treatment strategies. Patients diagnosed with *EGFR*-mutant lung cancer are routinely treated with one of the FDA-approved small molecule *EGFR* tyrosine kinase inhibitors (*EGFR* TKIs), erlotinib, gefitinib, or osimertinib. When applied, *EGFR* TKIs suppress *EGFR* phosphorylation and downstream activation of proliferative/prosurvival pathways involving MAPK, Akt, and STAT3. Initial tumor response to *EGFR* TKIs is frequently followed by development of treatment-tolerant and eventually treatment-resistant disease [68]. Some of these therapy-refractory tumors exhibit features of epithelial to mesenchymal transition (EMT) [69–72].

EMT is a process that drives epithelial cells to adopt a more invasive mesenchymal phenotype. Molecularly, EMT involves downregulation of epithelial marker

*CDH1* (encodes E-cadherin) and upregulation of mesenchymal markers, including vimentin (VIM), SNAIL, and ZEB1. In lung cancer models, EMT confers resistance to key treatment modalities including radiation [73,74], chemotherapy [75], and targeted therapeutics [69,76,77]. It has been shown that 1,25(OH)<sub>2</sub>D<sub>3</sub> opposes TGFβ-driven induction of EMT in lung epithelial cells, lung fibroblasts, and lung cancer cells [40,78]. Specifically, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment decreases TGFβ induction of an EMT gene expression profile, cell migration, and cell invasion. In preclinical *EGFR*-mutant, EMT-driven models of *EGFR* TKI tolerance, 1,25(OH)<sub>2</sub>D<sub>3</sub> not only restores expression of epithelial markers, but also promotes cell cycle arrest and improves sensitivity to *EGFR* TKIs [44].

EMT appears to be a common mechanism underlying emergence of lung cancer refractory to targeted therapies. NSCLC patients whose tumors are positive for rearrangement of the anaplastic lymphoma kinase (ALK) follow similar clinical course of disease progression in response to ALK TKI treatment to that of *EGFR*-TKI treated patients: initial tumor regression is usually followed by relapse under targeted therapy. A fraction of ALK-positive tumors with acquired TKI resistance undergoes morphological and molecular changes consistent with EMT [79,80]. Since the recent report suggests that the ability of vitamin D to promote sensitivity to targeted therapeutics may be linked to its proepithelial properties [44], vitamin D–based combination treatments may be beneficial for the patients with EMT-associated resistance to ALK TKI. This hypothesis requires further investigation.

Although the idea of exploiting vitamin D signaling to overcome EMT-associated TKI failure is intriguing, several studies demonstrate that reciprocal regulation occurs between 1,25(OH)<sub>2</sub>D<sub>3</sub> and EMT mediators (reviewed in Ref. [81]). Thus, while 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling opposes EMT, promesenchymal transcription factors such as SNAIL repress VDR expression [82,83]. The direct ability of promesenchymal transcription factors to repress VDR in NSCLC cells has not been tested. However, analysis of gene expression profiles from 75 NSCLC cell lines identifies a statistically significant inverse correlation between VDR and *ZEB1* [40]. Nevertheless, it has been demonstrated that vitamin D signaling axis remains intact and functional in *EGFR* mutant NSCLCs that underwent EMT while treated with *EGFR* TKIs [44]. Therefore, the opportunity exists to employ vitamin D–based therapeutics in the setting of progressive disease.

Another consistent mechanism that allows lung tumors to escape targeted therapeutics is MET amplification [14]. MET is a receptor tyrosine kinase situated upstream of PI3K/AKT and other oncogenic signaling cascades [14]. MET is activated by a pleiotropic factor

**TABLE 90.2** Stage- and context-specific actions of vitamin D in NSCLC.

Disease stage/ type	Treatment modality	Comment
Early stage	Surgery	Vitamin D3 supplementation improves RFS and OS among individuals with LUAD who are vitamin D deficient.
Advanced KRAS mt LUAD	Cytotoxic chemotherapy	Vitamin D3 metabolites unlikely to be effective. Tumors tend to be VDR low/CYP24A1 high and intrinsically refractory to vitamin D.
Advanced KRAS mt LUAD	Immune checkpoint blockade	Vitamin D3 metabolites may be effective. T cells express VDR. Vitamin D may reduce expression of immune checkpoints on T cells, prevent their exhaustion, and increase antitumor immunity.
Advanced EGFR mt LUAD	EGFR TKIs	Vitamin D3 metabolites may be effective. Tumors tend to express VDR. VDR signaling maintains tumor cells in an epithelial state in which they are sensitive to EGFR TKIs.

HGF and promotes survival, proliferation, and motility of tumor cells. Early studies conducted in human lung fibroblast cell lines reported modest but significant inhibition of HGF production in response to 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment [84]. Therefore, vitamin D may promote sensitivity or delay resistance to MET TKIs by modifying MET/HGF signaling.

Molecular profiling of lung tumors led to a fundamental shift in treatment paradigm of this deadly malignancy. Genotype-matched medicine is now a standard of care for many patients diagnosed with advanced lung cancer. Today, we recognize that treatment selection for these patients is best guided by targeting tumor-specific molecular alterations. Similarly, an accumulating body of evidence advocates for variable response to vitamin D in different tumor genotypes (Table 90.2). Thus, future research aimed to determine clinical utility of vitamin D in lung cancer can only benefit from careful refinement of tumor subsets considered for studies.

#### 5.4 Combining vitamin D with immunotherapeutics

For a select patient population diagnosed with NSCLC, immunotherapy has become a standard of care (Fig. 90.2). Several immunomodulatory agents approved for the use in advanced lung cancer target

the interaction between programmed cell death receptor 1 (PD1) on the surface of T cells and programmed death ligand 1 (PD-L1) expressed by the tumor cells. This interaction suppresses tumor reactivity of T cells, allowing cancer cells to expand and metastasize [85]. Tumor expression of PD-L1 is one of the biomarkers used to select patients who are likely to benefit from one of the immunotherapeutic agents [86]. Vitamin D has been shown to induce expression of PD-L1 in myeloid and epithelial cells [87]. Hence, combining vitamin D with immune checkpoint inhibitors may augment the efficacy of this class of drugs. Efficacy data should be available soon, as the effect of vitamin D<sub>3</sub> supplementation on treatment outcomes among patients with advanced cancer who receive immunotherapy is under active clinical evaluation (PROVIDENCE study) [88].

One could argue that potentially elevated levels of PD-L1 in response to vitamin D treatment may cause protumorigenic effects in patients who are not treated with immunotherapies. A recently published study by Li et al. [20] alleviates this concern. Patients with advanced NSCLC received chemotherapy with docetaxel followed by rocaltrol (0.5–2.0 µg/day) for 3 weeks. Controls received docetaxel alone. Blood was collected pretreatment and at conclusion of therapy for analysis of T cell biomarkers. At baseline, serum levels of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> were negatively correlated with immune checkpoints PD-1, TIGIT, and Tim-3 but positively correlated with expression of costimulatory molecule, CD28. Rocaltrol administration resulted in a significant increase in serum 1,25(OH)<sub>2</sub>D<sub>3</sub> and a significant decrease in PD-1, TIGIT, and Tim-3. Chromatin immunoprecipitation experiments showed that VDR binds to the promoters of these genes. In vitro treatment of T cells with 1,25(OH)<sub>2</sub>D<sub>3</sub> led to enhanced killing of tumor cells. In total, findings are consistent with a role for 1,25(OH)<sub>2</sub>D<sub>3</sub> (as a single agent) in directly repressing expression of immune checkpoints on T cells and promoting antitumor immunity.

It should be noted here that the efficacy of immune checkpoint inhibitors in *KRAS* mutant LUAD is well established [89]. Although tumor-intrinsic vitamin D signaling may not be effective in such tumors as discussed in Section VI A, the ability of vitamin D to regulate T cell function may nonetheless be an important determinant of treatment efficacy. Thus, one must consider not only the tumor genotype but also the treatment type in ascertaining the potential of vitamin D to influence lung cancer outcomes (Table 90.2).

Another layer of complexity relates to possible effects of vitamin D on drug metabolism. Nivolumab is one of the most commonly used immune checkpoint inhibitors for the treatment of advanced lung cancer. As with many pharmacological agents, nivolumab exposure directly correlates with tumor response [90]. Intriguingly,



patients treated with nivolumab whose circulating 25(OH)D<sub>3</sub> is below 10 ng/mL have been reported to have a significantly lower serum nivolumab concentration than those whose circulating 25(OH)D<sub>3</sub> is above 10 ng/mL [91]. This finding highlights the potential role of vitamin D in drug metabolism and needs to be validated by larger studies.

## 6. Strategies to maximize vitamin D signaling in lung cancer

To realize antitumorigenic potential of 1,25(OH)<sub>2</sub>D<sub>3</sub>, supraphysiological concentrations may be required, which leads to hypercalcemic toxicity in vivo. To address this issue, one of the following routes can be taken. Since tumor activity of vitamin D is known to be inversely correlated with expression of *CYP24A1*, its direct inhibition may increase tumor exposure to active metabolite of vitamin D and promote its antiproliferative activity. Alternatively, structural analogs of vitamin D with reduced calcemic activity can be used to minimize toxicity. Finally, novel formulations and routes of administration could limit systemic exposure and maximize vitamin D delivery directly to the tumor cells. In this section, we will highlight current efforts to maximize vitamin D activity in lung cancer.

A variety of vitamin D structural analogs have been synthesized and used in preclinical and clinical studies. Some of the compounds have reduced calcemic side effects and are successfully used in the clinic to treat psoriasis, renal disease, and secondary hyperparathyroidism [92]. While the use of vitamin D analogs has been widely investigated in malignancies of breast, colon, prostate, pancreas, and other sites, only few studies examined their effects on lung cancer pathogenesis.

Maxacalcitol has antiproliferative activity in LSCC cells in vitro, with significant growth suppression detected at concentrations as low as 10<sup>-9</sup>M. A similar magnitude of growth inhibition was achieved with 10<sup>-8</sup>M 1,25(OH)<sub>2</sub>D<sub>3</sub> [93]. Antiproliferative and antimetastatic activity of maxacalcitol was also demonstrated using the Lewis lung carcinoma model. In vitro, maxacalcitol inhibited production of MMP2, MMP9, and VEGF. Furthermore, mice injected intravenously with Lewis lung carcinoma cells and then treated with maxacalcitol developed fewer lung lesions while maintaining normal serum calcium levels and body weight. In contrast, a 10%–15% decrease in body weight and increase in serum calcium concentration were observed in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mice [94].

PRI2205, another less calcemic vitamin D analog, exhibited modest antiproliferative activity in A549 LUAD cells. When tested in the mouse Lewis lung

carcinoma xenograft model, PRI2205 had tumor-suppressive effects similar in magnitude to 1,25(OH)<sub>2</sub>D<sub>3</sub>, but with a significant reduction in toxicity and calcemic activity [95]. The outcome of combining PRI2205 with imatinib mesylate (Gleevec) was investigated in the A549 mouse tumor xenograft model, but with limited success. Although the combination of vitamin D analog and tyrosine kinase inhibitor slowed down growth of the tumors by ~50%, body weight reduction by ~15% was also noted, indicating moderate toxicity [96].

Seocalcitol (EB1089) enhanced response to radiation in H460 and A549 NSCLC cells [97]. The reported mechanism of action in irradiated cells involves a seocalcitol-dependent switch from cytoprotective to cytostatic autophagy, accompanied by cell cycle arrest. The radiosensitizing effect of seocalcitol was dependent on VDR and functional TP53 and was not observed in normal bronchioepithelial cells or cardiomyocytes, indicating specificity toward cancer cells [97]. These compelling results are yet to be replicated in vivo.

As discussed earlier, vitamin D antitumor activity is limited by high local levels of *CYP24A1*. This observation led to a series of studies to determine the effects of 24-hydroxylase activity on 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated transcription and antiproliferative activity in lung cancer. H292 cells expressing high levels VDR were treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> plus CTA091 and subjected to microarray analysis [98] and growth assays [63]. CTA091 is a selective inhibitor of 24-hydroxylase [99]. CTA091 inhibits 24-hydroxylase at low nanomolar concentrations and does not itself regulate gene expression through the VDR [100]. CTA091 globally potentiated the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on gene expression [98]. In growth assays, CTA091 had no single agent activity. However, it potentiated the antiproliferative effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> [63]. The ability of CTA091 to block 1,25(OH)<sub>2</sub>D<sub>3</sub> catabolism in H292 lung tumor xenografts has also been investigated: [101] CTA091 administration resulted in a 1.6-fold increase in plasma 1,25(OH)<sub>2</sub>D<sub>3</sub> and a 2.6-fold increase in intratumor levels of 1,25(OH)<sub>2</sub>D<sub>3</sub>. Compartmental modeling of 1,25(OH)<sub>2</sub>D<sub>3</sub> concentration versus time data confirmed that 1,25(OH)<sub>2</sub>D<sub>3</sub> was eliminated from plasma and tumor; CTA091 reduced the elimination from both compartments, and the effect of CTA091 on tumor exposure was greater than its effect on plasma. In total, these data indicate that 24-hydroxylase restricts 1,25(OH)<sub>2</sub>D<sub>3</sub> levels and signaling in lung cancer cells, and that 24-hydroxylase selective inhibitors (such as CTA091) may be used to enhance the anticancer effects of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Most common routes of vitamin D administration that are expected to result in elevated lung tissue exposure include dietary supplementation and IV injection of



active metabolites. While dietary vitamin D is generally safe, pharmacological concentrations in the lung are not likely to be reached. On the other hand, administering  $1,25(\text{OH})_2\text{D}_3$  via IV route is predicted to achieve higher levels of the active metabolite in the lung, but systemic exposure that may lead to hypercalcemia is also more likely to occur. Alternatively, high local concentrations of  $1,25(\text{OH})_2\text{D}_3$  in the lung may be achieved by intrapulmonary administration. Horiguchi and colleagues delivered a range of  $1,25(\text{OH})_2\text{D}_3$  doses (0–10  $\mu\text{g}/\text{kg}$ ) twice a week for 3 weeks directly into the airways of mice [102]. None of the dosing regimens resulted in significant loss of body weight. Bianchi et al. exposed mice to aerosolized  $1,25(\text{OH})_2\text{D}_3$  at 10ng/1 mL or saline for 10 min every other day for the duration of the study [103]. No change in body weight was reported. Furthermore, mice treated with aerosolized  $1,25(\text{OH})_2\text{D}_3$  developed significantly fewer lung tumor nodules derived from intravenous injection of colon cancer cells. Although the levels of  $1,25(\text{OH})_2\text{D}_3$  in the lung tissue and in the circulation were not evaluated in the aforementioned studies, no significant change in body weight was observed in all animals, suggesting lack of hypercalcemia-related toxicity. Detailed studies of tissue distribution of  $1,25(\text{OH})_2\text{D}_3$  following intrapulmonary administration are needed to evaluate clinical relevance and potential advantages of this vitamin D delivery method.

Finally, alternative formulations of vitamin D can be used to facilitate its delivery directly to tumor cells while reducing its availability to normal tissues. Nanoparticles are a novel platform for drug encapsulation that promises tumor delivery of its cargo with enhanced precision [104,105]. Hence, nanosystems may be particularly suitable for the use with vitamin D-based combination treatments. Few studies looked into employing this approach to deliver vitamin D to the tumors of the lung. In vitro, EGFR-targeted lipid-based nanoparticle (EGFR-LP) was used to codeliver  $1,25(\text{OH})_2\text{D}_3$  and 24-hydroxylase inhibitor CTA091 to EGFR-mutant TKI-resistant lung cancer cells [106]. Nanoparticles were taken up by the cells and induced greater vitamin D-dependent transcriptional response than the combination of free  $1,25(\text{OH})_2\text{D}_3$  and CTA091 at identical concentrations. Additionally, EGFR-LP-CTA091-VD promoted anticlonogenic activity of EGFR TKI erlotinib and increased expression of epithelial marker E-cadherin in the EMT-associated model of resistance to EGFR TKIs. As discussed before, CTA091 has been shown to augment antitumor activity of vitamin D. However, administration of free CTA091 is expected to result in systemic inhibition of 24-hydroxylase and hypercalcemia [100]. Therefore, the use of nanocarrier to encapsulate  $1,25(\text{OH})_2\text{D}_3$  and CTA091 is a compelling option to maximize

therapeutic efficacy of vitamin D. Follow-up in vivo studies are needed to evaluate the safety and efficacy of this novel vitamin D formulation. Although studies of vitamin D containing nanoparticles conducted in animal lung tumor models are lacking, in a murine model of breast cancer, pH-responsive nanoparticles carrying  $1,25(\text{OH})_2\text{D}_3$  in combination with paclitaxel (PCDMs) were found to be less calcemic than treatment with free  $1,25(\text{OH})_2\text{D}_3$  plus paclitaxel [107]. Significant increase in tumor accumulation of  $1,25(\text{OH})_2\text{D}_3$  in mice treated with nanomedicine versus free drugs was reported. This was accompanied by reduction in primary tumor volume and number of metastatic nodules in the lungs of the animals. The aforementioned results provide support for further investigation of safety and clinical utility of vitamin D-based nanomedicine for the treatment of lung cancer.

## 7. Conclusions

Promising signs of vitamin D activity in discreet subsets of patients with lung cancer are emerging. However, the full potential of vitamin D has yet to be realized. Precision clinical trials in which vitamin D supplementation is tested in combination with genotype-directed therapies among individuals with baseline deficiency are urgently needed. Precision trials should include tailored correlative studies, so that the underlying mechanisms (such as reversal of EMT or improved T cell effector function) can be confirmed. Parallel advances in the development of novel  $1,25(\text{OH})_2\text{D}_3$  analogs and  $1,25(\text{OH})_2\text{D}_3$  delivery systems are occurring. These formulations have potential to provide improved safety and efficacy and open new avenues of investigation in lung cancer.

## 8. Summary points

1. Preclinical studies demonstrate the ability of vitamin D to reduce the number and progression of premalignant airway epithelial lesions. Although no consistent association between vitamin D status and lung cancer risk has been established in the clinic, it appears that protective effect of vitamin D may be limited to populations with specific demographic characteristics, such as smoking status and BMI.
2. Similarly, in the setting of established disease, vitamin D is unlikely to be universally beneficial for recurrence-free or overall survival of lung cancer patients. Rather, in line with a contemporary precision medicine approach, vitamin D supplementation should be targeted to the most

responsive subtypes of lung tumors, including those with EGFR mutations.

3. Ongoing development of less calcemic vitamin D formulations and delivery systems may provide a safer way to implement high-dose vitamin D-based therapies for lung cancer treatment.

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## Further reading

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## Vitamin D and prostate cancer

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### OBJECTIVES

- Present the key data on prostate cancer incidence, mortality, and associations with 25-hydroxyvitamin D status.
- Discuss relevance of the dual disparities in African American men for prostate cancer and 25-hydroxyvitamin status.
- Present experimental evidence and clinical trials for a role for vitamin D metabolites in prevention and treatment of aggressive PCa.
- Describe genomic actions of vitamin D metabolites in prostate cells.
- Summarize current state of the field and discuss future directions.

### 1. Introduction: prostate cancer is a heterogeneous disease

In the United States, prostate cancer (PCa) is the most commonly diagnosed cancer in men with an estimated 268,490 new cases for 2022 (Cancer Facts & Figures 2022, American Cancer Society (ACS)). Globally, in 2020, approximately 1.4 million cases were recorded, and 375,000 deaths were attributed to PCa [1]. Within the United States, PCa has a 5-year survival rate of 98%, and this cancer remains the second leading cause of cancer deaths in men, with 34,500 deaths estimated for 2022. As these data illustrate, although PCa diagnosis is quite common worldwide, about 12% of US patients

ultimately experience a lethal form of PCa, whereas globally, mortality rises to 26%. These data indicate that the course of diagnosis and impact of treatment have divergent outcomes. The diversity of these outcomes includes the range of healthcare provision in different countries and the contribution of genomic ancestry [2].

PCa arises from a spectrum of molecular drivers that include those identified in precancerous conditions such as high-grade prostatic intraepithelial neoplasia (HGPIN). Most commonly, PCa is diagnosed when the cancer is localized to the prostate gland. Progression to invasive and metastatic disease can occur when patients experience therapy failure in response to curative attempts with either surgical or radiation approaches for localized disease. This invasive disease is then treated with systemic therapies, and ultimately androgen deprivation therapy (ADT) approaches, which are initially highly effective, but also have, ultimately, a high failure rate. This ADT-resistant form of PCa is typified by tissue plasticity and the development of so-called androgen-independent states. This final disease state is frequently lethal.

In parallel to this spectrum of disease states, with potentially differing therapeutic vulnerabilities, the pathological scoring system for PCa has changed over the years. Initially, grading relied on the Gleason grade for defining tumors; the Gleason grade characterized the aggressiveness of the tumor [3]. Thus, tumor foci with a Gleason grade  $\leq 3$  were characterized as low grade and those  $\geq 4$  as high grade. Subsequently, the concept of the Gleason score arose, as the sum of most common foci, and currently, this has been replaced by the Grade Group, which combines Gleason score with stage [4].



Nonetheless, uncertainties remain in prognosis despite these improvements in diagnosis accuracy. It remains unclear which men will experience treatment failure following curative attempts for localized disease. Similarly, in men with advanced disease, it is unclear which patients will experience a sustained tumor response toward to ADT, versus those for whom their tumor will rapidly progress.

The challenges of accurate PCa prognosis are amplified by a significant racial health disparity in which African American (AA) patients experience a more aggressive cancer that occurs at a younger age than in European American (EA) counterparts [5]; AA men experience a two-times risk of aggressive and lethal disease compared with their EA counterparts [6]. Indeed, AA men have higher prostate-specific antigen (PSA) [7] and larger tumor volume [8] compared with EA counterparts. Genomic ancestry underpins this disparity whereby genetic [9–11] and epigenetic [12–17] factors combine with biopsychosocial processes to drive AA PCa. For example, the incidence of *TMPRSS2* and *ETS* fusion genes [18,19] is common in EA PCa, but infrequent in AA counterparts [15,20,21]. As a result, the reasons for these disparities are multifactorial, likely involving biological factors as well as differences in lifestyle, screening, treatment, and quality of care in some cases. However, when controlling for socioeconomic factors and access to healthcare, the disparity persists with AA men presenting with PCa at a younger age and with disease that is more aggressive [22].

Set against this backdrop of a complex, heterogeneous, and evolving cancer, which is heavily impacted by genomic ancestry, a key challenge in the field of vitamin D research has been to identify in what PCa grade and progression context would vitamin D-centered therapeutic options be most effective (Fig. 91.1).

## 2. Epidemiologic associations between prostate cancer and vitamin D metabolites

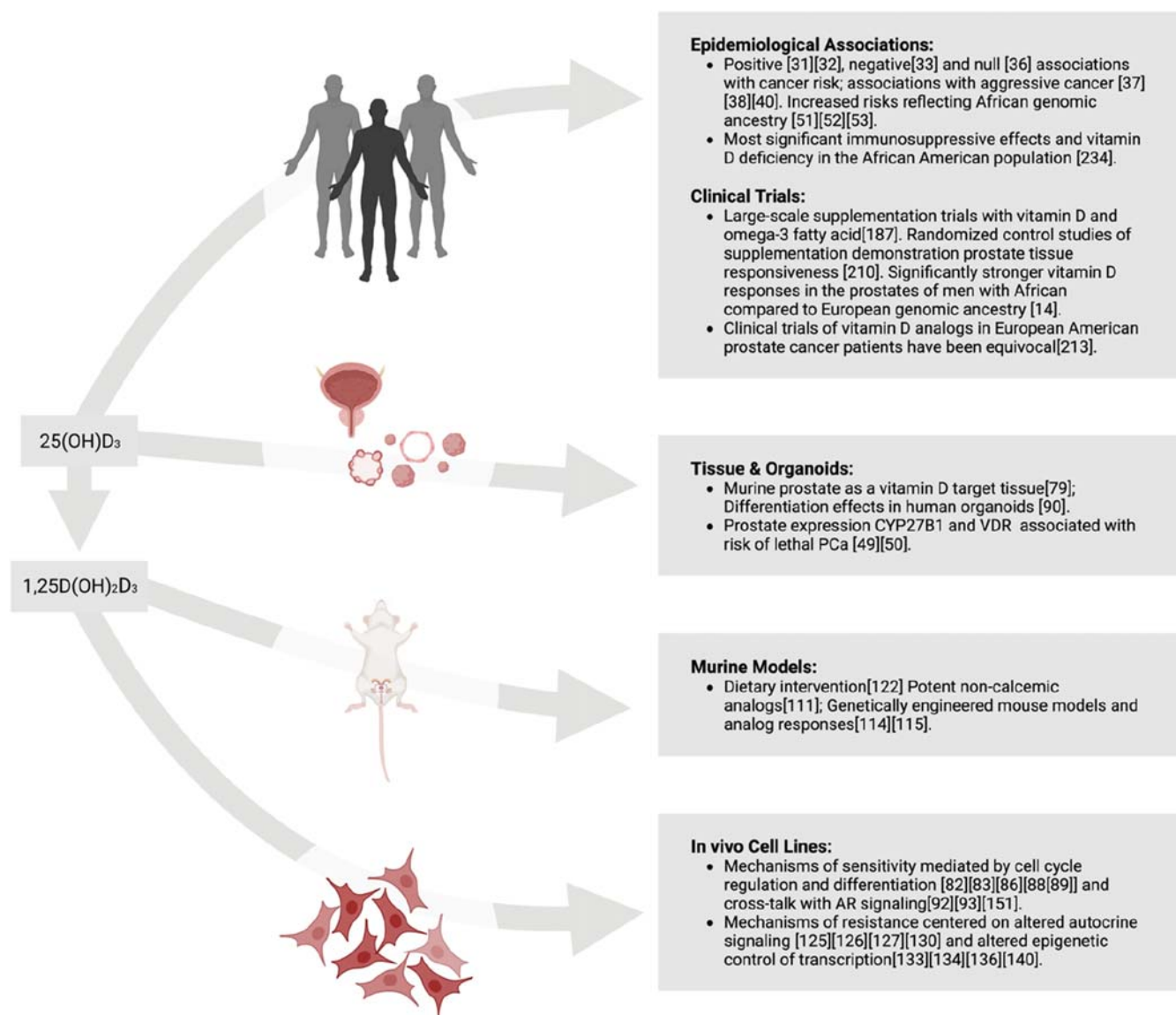
Epidemiological studies across several decades have analyzed blood levels of vitamin D metabolites and tested their associations with PCa risk and mortality. These studies began in the 1980s led by Cedric Garland and coworkers, who were largely responsible for initiating the analyses between solar UVB exposure, serum 25-hydroxyvitamin D (25(OH)D<sub>3</sub>), and cancer incidence [23,24]. These initial studies, which focused on colon cancer, were the catalyst for this team [25] and others [26–29] to investigate these relationships in the context of PCa.

The majority of PCa studies examine 25(OH)D<sub>3</sub>, but some include 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D<sub>3</sub> or calcitriol) and disease risk. When considering overall

PCa incidence, the results are mixed with both positive [30–32], negative [33,34], significant, and null [35–37] associations all reported, and therefore, it can be hard to make an overall interpretation. However, given the high prevalence of indolent PCa, one can argue that the association with risk of aggressive PCa has more clinical relevance, and there is a need to define the genomic characteristics when 25(OH)D<sub>3</sub> appears protective.

Studies that examine relationship between 25(OH)D<sub>3</sub> status and aggressive or lethal PCa overwhelmingly support that 25(OH)D<sub>3</sub> deficiency increases risk. A 2008 metaanalysis of seven cohort studies found a protective 0.91 hazard ratio (HR) for each 20 nmol/L increase in circulating 25(OH)D<sub>3</sub> [38]. Similarly, a prospective study of 1260 PCa patients and 1331 controls revealed that high serum 25(OH)D<sub>3</sub> associated with 57% reduction in risk of lethal PCa, but had no association with overall PCa risk [39]. An observational study in 2014 of ~3200 PCa patients and controls showed that increased 25(OH)D<sub>3</sub> serum levels were significantly associated with the reduced risk or higher grade PCa [40]. Similarly, a 2015 study using a smaller cohort attempted to test the significance of the association between circulating 25(OH)D<sub>3</sub> and risk of lethal PCa and but failed to identify significant relationships [41]. Possible explanations for these varied results are simple chance given the modest protective effects identified, disease severity, genomic ancestry of patients, diagnosis procedures, screening practices, and factors relating to vitamin D receptor biology that modify these associations [41]. For example, levels of vitamin D-binding protein (DBP) may modify the association between 25(OH)D<sub>3</sub> and risk of aggressive/lethal PCa [42].

Secondary analyses in large cohorts have also supported a chemopreventive role for 25(OH)D<sub>3</sub>. In the large Selenium and vitamin E Cancer prevention Trial (SELECT) of ~35,000 men, Kristal et al. observed a U-shaped association between serum 25(OH)D<sub>3</sub> levels and PCa risk overall as well as risk of aggressive high-grade PCa (Gleason score ≥7) [43]. As expected, the AA patients in this cohort had significantly lower serum 25(OH)D<sub>3</sub> levels than their EA counterparts, but associations with PCa risk could not be established as the AA cohort was much smaller than the EA one. Prior to SELECT, there were several smaller observational studies that also reported a U-shaped association between 25(OH)D<sub>3</sub> concentrations and PCa incidence [44–46]. Grant et al. reviewed U-shaped associations and posited that adverse risks at the higher concentrations may be due to inclusion of patients with underlying cardiovascular diseases and/or underlying pathophysiological processes altered by 25(OH)D<sub>3</sub>-induced changes in the immune system [47].



**FIGURE 91.1** Summary of the approaches to studying the vitamin D receptor system in prostate cancer. Investigators have applied different strategies from both observational and clinical trials in humans, to tissue isolates, murine models, and cell lines to consider the function of the VDR, 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> in the normal prostate and prostate cancer.

There are also significant associations between PCa risk and the expression of VDR-regulated genes. Epstein et al. showed that epidemiological studies of gene expression are unlikely to be biased by season of tissue collection as intraprostatic expression of vitamin D-regulated proteins (e.g., CYP27A1, CYP27B1, RXR $\alpha$ , CYP24A1) did not vary by season, in contrast circulating levels of vitamin D metabolites [48]. Such candidate gene studies can be challenging to undertake given the complex nature in which genes are regulated. However, it is interesting to note that low prostate expression of CYP27A1, a vitamin D-regulated gene, associated with higher risk of lethal PCa [49]. As well, tumor expression of the VDR protein itself within tumors

associated with reduced risk of lethal PCa with a striking HR of 0.17 in the Physicians Health Study and Health Professionals cohort [50].

The majority of epidemiological studies for PCa and vitamin D metabolites do not include racially diverse cohorts. However, those that do have shown that serum 25(OH)D<sub>3</sub> levels inversely associate with lethal PCa in AA men [51,52] and 25(OH)D<sub>3</sub> status predicted PCa biopsy outcomes [53]. Indeed, it has been suggested that only in AA men does the molar ratio of 25(OH)D<sub>3</sub>:1,25(OH)<sub>2</sub>D<sub>3</sub> associate with decreased risk of aggressive PCa [54].

Taken together, the epidemiological data consistently support an association between low circulating 25(OH)

D<sub>3</sub> and risk of aggressive/lethal PCa and that these associations appear to largest and most clinically impactful in AA men. However, these relationships are complex and reflect lifetime exposures, and controlling for life-style factors can significantly impact the strength of the relationships [55]. Larger studies may yield more refined thresholds for PCa risk as routine 25(OH)D<sub>3</sub> screening and supplementation have become more frequent in the past few decades, and indeed establish whether prostate-specific levels are most accurately established when based on genomic ancestry.

## 2.1 25-Hydroxyvitamin D status and the disparity of prostate cancer in men of African genomic ancestry

The importance of defining what is the correct 25(OH)D<sub>3</sub> status for prostate health, and how this varies by genomic ancestry and latitude, are key to determine how this signaling axis may prevent or reduce PCa risk. Reflecting the UVB-catalyzed synthesis of 25(OH)D<sub>3</sub>, skin melanin levels are a major predictor of 25(OH)D<sub>3</sub> deficiency [56]. Consequently, >90% of AA men have 25(OH)D<sub>3</sub> insufficiency according to current standards set by Hollis et al. [57,58], while 65% are considered deficient with serum 25(OH)D<sub>3</sub> levels below 20 ng/mL [59]. In comparison, ~60% of EA men have 25(OH)D<sub>3</sub> insufficiency, and only 20% are considered deficient [59]. Although the deficiency of 25(OH)D<sub>3</sub> in AA is undisputable, it is often considered a paradox, as at the population level the incidence of rickets or skeletomuscular disorders is not higher in the AA population. However, as discussed before, the threshold of 25(OH)D<sub>3</sub> concentrations for non-calcemic actions remains undetermined.

In regard to genomic ancestry and 25(OH)D<sub>3</sub> status, it is critical to discuss a highly publicized 2013 study by Powe et al., which demonstrated that AA men have lower circulating DBP; thus, although deficient in total 25(OH)D<sub>3</sub>, AA men have equivalent free-25(OH)D<sub>3</sub> [60]. However, it was quickly pointed out that this NEJM study utilized a monoclonal antibody ELISA, which has decreased affinity for Gc1F phenotype that is homozygous in >90s of African Americans, leading to underestimation of DBP in AA men [61,62]. Thus, throughout the chapter, we will include and highlight studies that examine genomic ancestry and/or utilize models from AA men.

## 3. The rationale to treat prostate cancer by targeting the VDR

Understanding the cellular impact of VDR signaling was profoundly catalyzed by studies in 1981 by Kay

Colston, led by David Feldman, who were the first to demonstrate that low nanomolar concentrations of 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the in vitro proliferation of human melanoma cell lines [63,64]. This was both a pioneering and fortuitous discovery. This was pioneering because it encouraged researchers to consider the impact of 1,25(OH)<sub>2</sub>D<sub>3</sub> on other cell systems [65–68], and also helped to illuminate the association studies undertaken by the Cedric Garland and coworkers the year before in 1980. These studies were fortuitous as the cells were obtained from a group in an adjacent laboratory who just happened to provide human melanoma cell lines. Thus, the discovery that 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the proliferation of cancer cells demonstrated that this seco-steroid hormone had functions in tissues that were not classical targets for the metabolism and deposition of calcium, and further this demonstrated the potential efficacy of 1,25(OH)<sub>2</sub>D<sub>3</sub> as an anticancer agent. It is interesting to note that this important work, and other analyses during the 1980s on 1,25(OH)<sub>2</sub>D<sub>3</sub> as an anticancer agent, preceded the cloning of the VDR in 1988 [69].

The importance of this work resonated with others who were examining other compounds that could induce cancer cell differentiation. For example, the impact of retinoids that target the retinoic acid receptors, a cousin of the VDR, was investigated to drive cell differentiation of HL60 leukemia cells [70]. In turn, these had studies led to the pharmacologic exploitation of all-*trans* retinoic acid in leukemia [71] and were the catalyst for the development of the concept of differentiation therapy [72–74]; all-*trans* retinoic acid remains a current clinical option in acute promyelocytic leukemia. It is within this context, therefore, that the potential for exploiting 1,25(OH)<sub>2</sub>D<sub>3</sub> as a potential differentiation agent was explored in a wide variety of cancer cell lines, including PCa, all major solid tumors and leukemia [75–77].

## 3.1 Effects of vitamin D and analogs in preclinical models of prostate

A large body of literature has investigated the cellular impacts of targeting the VDR by 1,25(OH)<sub>2</sub>D<sub>3</sub> and a number of pharmacological analogs [78]. An initial focus was to establish that the murine prostate was a possible vitamin D target tissue [79] and that prostate cancer cell lines expressed the VDR [80]. However, the first targeting of the VDR in human PCa cell lines was in 1993 and clearly established that while the three cell lines examined expressed comparable levels of VDR, there was no clear relationship to the cellular sensitivity toward 1,25(OH)<sub>2</sub>D<sub>3</sub>. For example, LNCaP and DU-145 cells expressed comparable amounts of VDR, but



although LNCaP was strongly growth inhibited by  $1,25(\text{OH})_2\text{D}_3$ , DU-145 cells were essentially resistant [81]. This initial and powerful study therefore established the questions of the field, namely: What was the molecular mechanism(s) that mediated sensitivity? What mechanism(s) led to  $1,25(\text{OH})_2\text{D}_3$  resistance?

### 3.1.1 *In vitro analyses of mechanisms that mediate $1,25(\text{OH})_2\text{D}_3$ sensitivity*

Reflecting the analyses of  $1,25(\text{OH})_2\text{D}_3$  actions in leukemia cells, many investigators examined how the cell cycle was  $1,25(\text{OH})_2\text{D}_3$ -regulated in prostate cells. These studies identified VDR regulation of genes that control cell cycle progression, including the cyclin-dependent kinase inhibitors, p21<sup>(waf1/cip1)</sup> and p27<sup>(kip1)</sup> [82], as well as the direct VDR-binding sites on the gene *CDKN1A* (encodes p21<sup>(waf1/cip1)</sup>) [83]. The  $1,25(\text{OH})_2\text{D}_3$  regulation of p27<sup>(kip1)</sup> appears to be mechanistically enigmatic and includes nondirect mechanisms [84,85]. Other genes identified to be direct VDR targets regulated by  $1,25(\text{OH})_2\text{D}_3$  and regulate cell cycle include GADD45 $\gamma$  [86] and IGFBP3 [87].

Again, perhaps less clear in terms of mechanism, but nonetheless biologically impactful, is also the  $1,25(\text{OH})_2\text{D}_3$ -mediated downregulation of MYC mRNA and protein [88]. This is accompanied by dephosphorylation of retinoblastoma protein allowing E2F family members to bind and downregulate cyclin A, ultimately sustaining G<sub>1</sub> cell cycle arrest [85]. Thus, the regulation of cell cycle progression appears to be a clear mechanism by which  $1,25(\text{OH})_2\text{D}_3$  signaling can reduce cell proliferation.

Across cell biology systems, a common theme is that cell cycle arrest is permissive of cell differentiation. For example, treatment of leukemia cells with retinoids, or  $1,25(\text{OH})_2\text{D}_3$ , clearly induces defined differentiation programs that are phenotypically distinct. This is far less clear with PCa cells, or even nonmalignant prostate cells. Various groups have suggested that  $1,25(\text{OH})_2\text{D}_3$ -induced expression of E-cadherin and PSA are concomitant with a more differentiated and adherent luminal cell phenotype [81,82,89], and similarly, there is evidence that  $1,25(\text{OH})_2\text{D}_3$  regulates prostate differentiation factor (PLAB), which is a member of the bone morphogenetic protein family required for growth and differentiation of both embryonic and adult tissues [87]. Direct regulation of individual differentiation genes by  $1,25(\text{OH})_2\text{D}_3$  has been shown in several models. McCray et al. showed accelerated differentiation by  $1,25(\text{OH})_2\text{D}_3$  in patient-derived benign prostate organoids from 11 patients and regulated the WNT pathway via suppression DKK3 [90].

One plausible mechanism by which the VDR may induce prostate differentiation is through cooperation with androgen activity through the androgen receptor

(AR). Certainly, AR is associated with potential prostate epithelial differentiation in a number of ways. First, temporal expression of AR and androgen cross-talk between epithelium and stroma is essential for normal prostate development [91], underscoring paracrine signaling and cross-talk during this process. Secondly, within the epithelium, from which PCa arises, only the secretory, luminal cell populations express AR. Thus, it may be that the VDR augments these actions, perhaps through shared target genes. More specifically,  $1,25(\text{OH})_2\text{D}_3$  induced AR expression in LNCaP, CWR22R, MDA-PCa-2a, and MDA-PCa-2b cell lines [92–96], and for example, LNCaP cells respond differently to  $1,25(\text{OH})_2\text{D}_3$ , depending on the presence of androgens, further suggesting interplay between these two hormones [80]. In the opposite direction, pharmacological inhibition of AR activity abrogated antiproliferative effects of  $1,25(\text{OH})_2\text{D}_3$  in LNCaP cells [95,97]. Although these studies in PCa cell lines support cross-talk between the hormones, functional differentiation was not demonstrated. Furthermore, androgens upregulate VDR expression [98], further supporting the communication between these two hormonal axes. Finally, in PCa patients, several studies have shown that vitamin D supplementation reduced PSA levels [99–101]. Together, this suggests there is a functional cross-talk between the AR and the VDR, which may coordinate cell fate decisions including differentiation.

Despite these mechanisms of potential cross-talk, and hints that the genes coregulated may impact cell lineage, it is unclear whether the actions of the VDR, including with the AR, are sufficient to drive functional luminal differentiation. For example, microarray or RNA-Seq analyses in prostate systems have not clearly established a significant change in luminal gene expression programs that are overtly implicated in differentiation. That is,  $1,25(\text{OH})_2\text{D}_3$  signaling drives a cell cycle arrest, but probably other signals may be required to induce differentiation [102–104] including regulation of WNT signaling [90].

More broadly, other aspects of  $1,25(\text{OH})_2\text{D}_3$  anticancer actions include the suppression of inflammation, inhibition of angiogenesis, reduction in invasion and metastasis, and induction of programmed cell death events in a variety of benign prostate epithelial and PCa cell lines [105–108].

### 3.1.2 *In vivo analyses of $1,25(\text{OH})_2\text{D}_3$ actions*

Given these significant insights into the potential anticancer actions of the VDR in PCa, there has been enthusiasm to exploit murine models to define either chemoprevention or chemotherapy strategies. However, a difficulty in undertaking in vivo studies is, of course, that there are species differences between mice and humans, and consequently the findings may need to



be interpreted cautiously. For example, these differences include that the longevity and the age-associated cancers of mice do not obviously reflect humans, and indeed, there are other key metabolic differences between the two species that may impact on how  $1,25(\text{OH})_2\text{D}_3$  impacts xenograft PCa cells, or genetically engineered mouse models (GEMM) of PCa [109,110]. The murine and human prostates are anatomically distinct, and therefore, how  $1,25(\text{OH})_2\text{D}_3$  impacts these tissues is unclear. Finally, the regime of testing is challenging to recapitulate human PCa, especially in the case of identifying which chemoprevention time windows are most effective. Finally, a crucial challenge for in vivo studies is the dose-limiting hypercalcemic toxicity of treating with  $1,25(\text{OH})_2\text{D}_3$ , and thus, noncalcemic analogs have also been developed with the goal to avoid this deleterious side effect [111,112].

Use of  $1,25(\text{OH})_2\text{D}_3$  and analogs to prevent and/or treat cancer of GEMM mouse models of PCa has in some ways recapitulated the findings in human PCa cell lines, but also yielded mixed results. Treatment of *Nkx3.1;Pten* mutant mice, a transgenic model for PCa, with  $1,25(\text{OH})_2\text{D}_3$  delayed the formation of HGPIN lesions [113]. However, there was no benefit to treating mice with  $1,25(\text{OH})_2\text{D}_3$  after precancerous lesions had formed. In a similar model of *Pten* mutation driving HGPIN, a recent study that used single-cell RNA sequencing (scRNA-Seq) revealed that although a potent noncalcemic analog of  $1,25(\text{OH})_2\text{D}_3$ , Gemeni-72, initially caused apoptosis of most of the HGPIN cells, the surviving ones became more aggressive and displayed upregulated survival pathways [114]. Similar results were observed in the TRAMP mouse, in which  $1,25(\text{OH})_2\text{D}_3$  initially inhibited growth of PCa, but the cells that escaped restraint appeared to be more aggressive and in fact the treated animals displayed an increase in metastatic cancer [115]. It is unclear if this finding recapitulates human PCa although the TRAMP model displays frequent disruption to p53 and Rb [116], which are also highly frequent events in human PCa [117].

Finally, it is interesting to note where the *Vdr*<sup>-/-</sup> mice have been informative in the cancer context. Specifically, crossing the *Vdr*-ablated background into models of tumor disposition phenotypes can reveal biological antagonism between *Vdr* and carcinogenic signals. For example, *Vdr*-deficient and heterozygote background mice exacerbated the aggressiveness of murine breast [118,119] and colon [120,121], but similar experiments have not been reported with well-established murine PCa models, although absence of proof is not proof of absence. However, deletion of *Vdr* in the TgAPT(121) mice (a model of prostate intraepithelial neoplasia) did result in a more aggressive cell state with less programmed cell death [122].

### 3.1.3 Mechanisms of resistance toward the anticancer actions of $1,25(\text{OH})_2\text{D}_3$

A significant focus from multiple investigators has been to identify the resistance mechanisms that suppress the cell responsiveness toward  $1,25(\text{OH})_2\text{D}_3$  and may therefore provide a rationale target for therapeutic cotargeting. A classical approach has been to consider the local autocrine signaling of VDR in the prostate.

In the prostate, there is evidence of extrarenal expression of *CYP27B1* enzyme indicating that local intraprostatic production of active  $1,25(\text{OH})_2\text{D}_3$  may regulate tissue levels of the active hormone [123–125]. PCa cells have decreased *CYP27B1*, thus likely reducing ability to form  $1,25(\text{OH})_2\text{D}_3$  [126,127]. Interestingly, studies have reported vitamin  $\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  are equally potent in vivo in terms of anticancer actions, supporting a route for local tissue activation [128]; however, reduced expression of *CYP27B1* is observed in PCa cell lines [127]. By contrast, *CYP24A1* is expressed in a frequently amplified region of Chr 20q13 and has putative oncogenic functions [129], and its expression is increased in PCa associated with loss of sensitivity toward  $1,25(\text{OH})_2\text{D}_3$  [130]. Of course, within a tumor microenvironment, the cross-talk with stroma cells can also impact these mechanisms, and there is evidence that VDR signaling in different cell populations needs to be considered [131].

A perhaps more widespread mechanism of disruption of VDR signaling in PCa is through epigenetic mechanisms and has been a subject of significant focus to understand they may disrupt VDR signaling in terms of the ability of the VDR transcriptional complex to function. The rationale for considering that the VDR transcriptional functions are distorted is apparent from considering cells such as DU-145, which are essentially resistant to the anticancer actions of  $1,25(\text{OH})_2\text{D}_3$  [81], but still respond transcriptionally, albeit in a selectively distorted manner, such that classical antiproliferative target genes such as *CDKN1A* are not regulated, whereas the upregulation of the catabolic *CYP24A1* gene is retained [132–134]. These findings demonstrate that a lack of functional VDR alone, or ability to initiate catabolism alone cannot explain resistance, and also support the concept that the VDR transcriptome is altered in cancer cells to disfavor antiproliferative target genes.

One approach has been to examine how CpG methylation patterns impact VDR signaling. In other cancer settings, DNA methylation has been associated with the disruption of expression of the VDR itself [135], and within PCa, altered expression of the *CYP24A1* gene appears to be driven also by changes in DNA methylation [136,137]. Furthermore, the VDR can downregulate DNMTs, leading to gene-specific hypomethylation and resistance to  $1,25(\text{OH})_2\text{D}_3$  [138].

Other studies have pursued how the interactions of VDR with transcriptional corepressors such as NCOR1, NCOR2/SMRT, and LSD1 may be distorted in PCa have been examined to investigate this possibility [133,134,139–143] and also that cross-talk with AR, and its coregulators may also distort VDR functions [144]. In turn, it has been suggested that this enhanced interaction with corepressors presents a therapeutic vulnerability that can be exploited by treating cells with 1,25(OH)<sub>2</sub>D<sub>3</sub> plus HDAC inhibitors [133,134,139, 145,146].

## 4. High-dimensional data approaches to understanding VDR functions in the prostate

### 4.1 Transcriptomic approaches

Gene microarray and subsequently RNA-Seq approaches are the earliest and most widespread high-dimensional approaches applied to prostate systems. Some genes and networks are consistently identified across experiments, but there is much diversity, reflecting different cells examined, time and dose of treatment, and different microarray platforms used with differing numbers of genes, as well as distinct downstream analytical approaches.

In 2003, one of the first microarray analyses in PCa cells (LNCaP) confirmed the *CYP24A1* was a regulated gene target, as was *IGFBP3* but also identified changes in expression of *MAPK* as well as genes such as thioredoxin reductase 1, involved in redox balance, and a number of metallothioneins that are involved in the inhibition of programmed cell death [147]. In the same year, others identified enrichment of genes that were directly regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR including metabolic enzymes and others involved in the control of fatty acid biosynthesis [148]. Interestingly, in the latter case, this involved genes being directly transrepressed by the VDR, which remains mechanistically enigmatic.

Following on from these initial studies, others [133] examined the context of 1,25(OH)<sub>2</sub>D<sub>3</sub> resistance and the use to HDAC inhibitors to identify regulation of genes that could be restored in PC-3 that were essentially recalcitrant to the actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> alone. This approach also identified signal transduction genes such as *MAPKAPK2* and *GADD45α* reflecting the findings of others, but also identified novel genes including DNA-binding protein inhibitor *ID-1*, which can inhibit a wide variety of basic helix loop helix transcription factors, and perhaps helps to explain the pleiotropic actions of 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR signaling. Extending the concept that 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR may regulate transcriptional mechanisms further was the identification in ALVA-31 cells that the genes regulated at mRNA and protein level

included estrogen receptor alpha, and heat shock proteins suggesting that VDR activation will directly impact how estrogen and androgen will signal in the system, and supporting a cross-talk between these different classes of nuclear receptors [149]. Indeed, this concept has been followed up by a number of investigators, for example, who reported microarray-identified regulation by 1,25(OH)<sub>2</sub>D<sub>3</sub> of AR target genes such as *TMPRSS2* and downregulation of *MYC*; given that *MYC* is a basic helix loop helix transcription factor, it is interesting to speculate that this may reflect the impact of upregulated *ID-1* [150].

The concept of 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR and DHT/AR cross-talk was previously introduced. This cross-talk was further explored by Tenniswood and coworkers who examined the cellular and transcriptional impact of the cotreatment in LNCaP cells [151]. These workers demonstrated cooperative actions on cell proliferation as well as a unique gene set of ~250 genes in the dual treatment. Gene set enrichment identified in upregulated gene ontologies enriched for ion transport, for example, and in the downregulated gene ontologies that included DNA repair. Validation studies confirmed the combinatorial impact on a number of genes including cell cycle genes such as *GADD45G* and *CDC20* as well as others in lipid metabolism such as *HPGD*. These workers also undertook the interesting step of undertaking miRNA microarrays to identify miRNA in inverse correlation with mRNA that could explain the regulation of the observed mRNA. For example, 1,25(OH)<sub>2</sub>D<sub>3</sub>/VDR and DHT/AR downregulated miR-17, which was inversely correlated with the upregulation of a number of genes that block apoptosis. The cross-talk of VDR regulated miRNA and mRNA in incoherent feed-forward loops has been established at the candidate level in nonmalignant prostate epithelial cells [83]. Finally, the regulation of miRNA by the VDR has been examined further, for example, to consider how VDR-regulated miRNA in stromal cells impacts the tumor [152], and identified direct roles for VDR regulation of DICER to explain in part the regulation of miRNA. Others have reported significant diversity in 1,25(OH)<sub>2</sub>D<sub>3</sub> regulation of miRNA across prostate cells [153]. Indeed, the significance of the differences in expression of miRNA in different cells supports a role for secreted miRNA to be prognostic of aggressive PCa, which has been validated by various workers [154].

### 4.2 Cistromic approaches

With the development of chromatin immunoprecipitation coupled with NGS (ChIP-Seq), it has been possible to map the genomic locations of the VDR in a

variety of cell systems [155–159]. To date, there are only three published datasets of VDR ChIP-Seq in prostate cells ([160,161], doi: <https://doi.org/10.1101/2022.01.31.478573>), and one publicly available dataset in LNCaP (GSE64657), but which has not yet been analyzed and published.

Potentially, VDR ChIP-seq studies carry more biological importance than transcriptomic approaches as they establish direct VDR genomic interactions, compared with the combination of direct and indirect actions that are represented by transcriptomic approaches. By contrast, annotation of ChIP-Seq data remains a more complex bioinformatic procedure than transcriptomic approaches as the challenge of establishing meaningful *cis-trans* relationships is a significant one [162,163]. A given cistrome needs to be defined in genomic space, for example, with ChromHMM algorithm [164] to define underlying epigenetic states, or GIGGLE to define overlapped with other cistrome data [165], or the ROSE algorithm to define superenhancers [166,167]. Next, these parsimonious cistrome definitions of the unique and overlapping genomic distributions in different epigenetic states (e.g., in active enhancers, at poised enhancers) in cells/treatments need to be integrated with matched RNA-Seq undertaken in parallel treatments to define cistrome–transcriptome relationships and test their phenotypic associations, for example, using Kolmogorov–Smirnov tests to examine differences in cumulative distribution plots for cistrome-binding sites with respect to nearest gene, using bootstrapping approaches to measure how the specific cistrome relationships associate with gene expression patterns [153]. Needless to say, this can result in considerable variation in the findings generated.

Work by Fleet et al. [160] identified VDR binding at ~3400 protein-coding genes, ~680 long noncoding RNAs, and ~470 miRNAs, which included VDR-bound peaks at known VDR target genes including *CYP24A1* and *IGFBP3*. Interestingly, peak distribution was evenly divided between intergenic and intronic regions, supporting both long-range and proximal regulation. These studies also suggested that  $1,25(\text{OH})_2\text{D}_3$  amplifies signals mediated through other TFs including NF-kappa-B inhibitor alpha (NFKBIA) and FOXO1, and some peaks near immune response-related genes (e.g., *L1R2*) hint toward VDR regulation of immune processes.

These data were also reflected in a VDR-ChIP Seq study in nonmalignant PrE cells [161], which identified VDR-binding sites, again including in well-known targets (e.g., *CYP24A1*) and, interestingly, ligand activation led to a significant decrease in the number of VDR-ChIP peaks, reflecting perhaps an active role for the basal VDR in gene expression. Also of interest in this study was the identification of significant sites where there

was a loss of VDR binding, for example, at the aminoacyl tRNA synthetase genes, which led to decreased proliferation. Interestingly, VDR was also identified to bind near genes regulating neural differentiation, which raises an interesting possibility that VDR genomic functions may be linked to neuroendocrine transdifferentiation in PCa, which occurs in advanced ADT-resistant PCa.

Finally, a recent study [168] addressed VDR function in the context of PCa health disparities by examining a panel of EA cells (HPr1-AR and LNCaP) and AA cell lines (RC43N, RC43T, RC77N, and RC77T). These analyses revealed that the VDR genomic interactions were significantly more frequent and stronger in transcriptional regulator in AA than EA prostate cells and that in PCa this signaling is distorted and suppressed. In nonmalignant RC43N cells, VDR ChIP-Seq identified significant basal and  $1,25(\text{OH})_2\text{D}_3$ -dependent VDR-binding sites, with ~1300 in total associated with transcriptional responses enriched for circadian rhythm and inflammation networks. In parallel,  $1,25(\text{OH})_2\text{D}_3$ -dependent ATAC-Seq also revealed the greatest impact on chromatin accessibility in RC43N cells, with a significant gain of nucleosome-free regions at enhancers. By contrast, in EA and AA PCa cell models,  $1,25(\text{OH})_2\text{D}_3$  led to a loss of VDR binding. Coupled with these different cistromic patterns, proteomic analyses also suggested that genomic ancestry significantly impacted the protein partners within the VDR complex.

Other studies either outside of prostate biology, or within the prostate but not directly examining the VDR, have revealed two other aspects of how the VDR may impact the prostate. Firstly, an obvious concept is how genetic variation impacts VDR function. An initial focus on understanding how VDR function in cancer could be disrupted was to consider candidate SNPs in the receptor itself, which could be characterized by restriction enzyme digestion. This led to the possibility that specific VDR-associated SNPs may be associated with altered VDR function [169]. These findings were initially suggestive of a functional relationship between VDR genetic variation and cancer risk. However, none of these associations are genome-wide significant, and indeed, there are no SNPs in the National Human Genome Research Institute genome-wide association studies (GWAS) catalog that annotates VDR to prostate phenotypes.

An alternative impact of genetic variation is on the sites of VDR binding. Given that more than 90% of human genetic variation is in noncoding regions of the genome, it is not unsurprising that VDR-binding sites may overlap with GWAS SNPs. This is a challenging computational analysis, and several approaches have been developed [170]. Within the context of VDR, VDR cistrome data have been overlapped with GWAS SNPs,



and SNPs in linkage disequilibrium, to test the interaction between disease- and phenotype-associated SNPs at sites of VDR genomic binding. These analyses identified where GWAS SNPs were significantly enriched in VDR cistromes, and for example, identified a significant overlap between VDR and nuclear factor- $\kappa$ B binding sites that contained GWAS SNPs related to immunophenotypes [171]. Given that the number of prostate-specific VDR cistromes is increasing as are the number of studies with GWAS SNPs associated with PCa [10,172–178], revisiting this approach will potentially reveal how germline genetic variation, including as a result of different genomic ancestry, impacts the ability of the VDR to bind and regulate target genes.

Secondly, genomic approaches are beginning to annotate VDR cistromes to address the question of what transcription factors or coregulators are shared at the sites VDR binding. Initial candidate studies of VDR binding suggested that there the specificity of binding for different nuclear receptors was dictated by the nucleotide spacing between hexameric motifs [179]. This very elegant idea has largely been unsubstantiated by cistromic data, and there appears to be a genuine knowledge gap in determining how the specificity of nuclear receptor binding arises. Most likely insights from the 3D genome [180], phase condensates [181–183], and protein–protein interactions will yield significant insights. Indeed, a recent analyses of how VDR functions to form topologically associated domains suggest that the VDR plays a significant role in this capacity [180] and will most likely be highly impactful in considering the function of this receptor in cancer.

Finally, it is also perhaps not surprising that the VDR, as a type II nuclear receptor and being nuclear resident independent of ligand, impacts transcriptional process through a range of mechanisms and indeed recently a so-called bookmarking functions for the VDR have been identified [184]. Within this context of VDR protein–protein interactions, it is interesting to note the corepressor NCOR2 significantly overlaps with VDR motif and cistrome in ADT-resistant PCa cell models, suggesting the VDR functions are suppressed with PCa progression [185] and may include the ability of the VDR to bookmark for other nuclear receptors.

## 5. Lessons from PCa clinical trials with vitamin D compounds

The epidemiological relationships between vitamin D metabolites and risk of aggressive PCa combined with anticancer effects of  $1,25(\text{OH})_2\text{D}_3$  in preclinical models paved the way for clinical trials. Indeed, there has been an active portfolio of clinical trials in the arena of

vitamin D supplementation or treatment with  $1,25(\text{OH})_2\text{D}_3$  PCa for many years considering different aspects of chemoprevention and chemotherapy.

### 5.1 Chemoprevention of PCa by vitamin D supplementation

Epidemiologic studies have shown positive, negative, U-shaped, and null associations between PCa risk and vitamin D metabolites, as detailed before. In an effort, in part, to address these ambiguities, several large-scale randomized supplementation trials are ongoing including the VITAL (VITamin D and omega-3 TriaL), which has accrued  $\sim 25,000$  people and is examining the impact of supplementing vitamin D and omega 3 fatty acid on a range of pathologies, cancer, and heart disease incidence [186]. This 5-year study was randomized, double-blind, placebo-controlled  $2 \times 2$  trial with 2000 IU/d of cholecalciferol, and 1 g/d of marine omega-3 fatty acids in 25,871 men over 50 and women over 55 years of age. There principal results were reported in 2020 for the primary endpoints of cardiovascular disease and cancer risk [187]. The overall incidence of PCa during the trial was 192, with an HR of 0.88 (0.72–1.07) for combined intervention and 1.09 (0.73–1.62) for the vitamin D intervention arm, both of which were not different from placebo. The HR for confirmed metastatic PCa or PCa-specific mortality was 0.43 (0.17–1.12) although also not significant with only 20 total events. When examined by race, AA men had lower HRs for PCa in vitamin D intervention arm, although not significant due to a very low number of events [188].

Although, at first glance, the VITAL results appear to show no benefit to vitamin D supplementation and PCa risk, it is important to consider several factors that impact the results. The first is that there were very few cases of metastatic PCa in the cohort overall, with only 20. It would be nearly impossible to see a benefit with these small numbers, but the results strongly trend in a direction that vitamin D is protective. Secondly, the intervention time of 5 years is very short in relation to a lifetime of vitamin D deficiency. The molecular events leading to carcinogenesis accumulate over a lifetime, and mutations are irreversible. One would only expect a benefit from a short intervention if vitamin D was able to impact the PCa lesions themselves and alter progression. In general, RCTs are designed to test therapeutics with S-shaped efficacy curves and therefore frequently fail for supplementation studies [189]. Another limitation is that we do not know the threshold of  $25(\text{OH})\text{D}_3$  levels needed for nonskeletal effects, which may result in insufficient dosing in RCTs. There are also polymorphisms in vitamin D–related genes that likely



contribute to heterogeneity of response and overall serum 25(OH)D<sub>3</sub> levels.

However, there are clues in this study that the associations between UVB radiation vitamin D<sub>3</sub> and PCa are more striking in AA men. Given that UVB radiation also degrades folic acid, a strong inverse correlation between skin pigmentation and latitude has arisen during ancestral adaptation [190,191]. Relatively, recent rapid dispersion and lifestyle changes have resulted in many individuals currently living in UVB environments that differ profoundly from their ancestral ones. Reflecting this, significant associations have arisen between low serum vitamin D<sub>3</sub> levels and cancer incidence and progression risks among AA PCa patients [51,53,59,192–203]. Furthermore, although vitamin D<sub>3</sub> supplementation in the VITAL cohort [204,205] had no overall impact on cancer incidence, the AA participants experienced a suggestive 23% ( $P = .07$ ) reduction in cancer risk, indicating that larger cohorts may be more informative [188,206,207].

Therefore, a second strategy has been to undertake randomized controlled trials (RCTs) in men with or without PCa using vitamin D supplementation. There are also studies that supplement PCa patients before radical prostatectomy, which have identified and validated molecular targets and pathways regulated by vitamin D metabolites. Cholecalciferol supplementation of men with PCa has shown changes in clinical aspects of the disease as well as molecular targets. In patients with recurrent PCa, cholecalciferol (2000 IU/day) significantly reduces PSA doubling time in a small pilot of 15 PC patients [101]. In a cohort of 44 men with low-risk PCa, 1 year of 4000 IU/d reduced the number of biopsies positive for PCa [208]. Wagner et al. conducted a double-blind, presurgical intervention in PCa patients who were treated with 400, 10,000, or 40,000 IU/d for 8–12 weeks. They observed no toxicity, and the prostate calcitriol levels negatively correlated with the number of dividing cells by Ki67 positivity [100]. The highest dose group also had lower PSA compared with the other dose groups. Using that same cohort, Giangreco et al. showed that the expression of inflammatory genes [124] and microRNAs [209] correlated with prostate 1,25(OH)<sub>2</sub>D<sub>3</sub>. Collectively, these studies demonstrate that prostate tissue is responsive to cholecalciferol supplementation and suggest suppression of inflammatory androgen pathways by cholecalciferol. However, none of these studies included a diverse population of patients.

To identify mechanisms of 1,25(OH)<sub>2</sub>D<sub>3</sub> sensitivity in PCa patients, a number of investigators have considered the option of treating men with localized disease before surgery and then examining the tumor material retrieved after surgery for analyses of VDR signaling pathways. For example, in a pilot study of 40 patients

with localized PCa, Beer and colleagues tested 1,25(OH)<sub>2</sub>D<sub>3</sub> or placebo for 4 weeks before radical prostatectomy [210]. Immunohistochemistry of VDR and known candidate VDR target genes coupled with markers of cell proliferation were examined confirmed 1,25(OH)<sub>2</sub>D<sub>3</sub> responses in the prostate tissue. These and other studies suggest that 1,25(OH)<sub>2</sub>D<sub>3</sub> could be given to PCa patients, even at quite high doses [211], and biological effects could be observed. Indeed, this led to the concept of combining 1,25(OH)<sub>2</sub>D<sub>3</sub>, or more stable formulations, with existing chemotherapies.

## 5.2 Chemotherapy of PCa with 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs and the impact of genomic ancestry

One such approach was undertaken by Novoclea who undertook a series of clinical trials with a 1,25(OH)<sub>2</sub>D<sub>3</sub> compound, DN-101, in combination with docetaxel in men with advanced prostate cancer who had experienced ADT treatment failure in a randomized phase III study (ASCENT I [AIPC Study of Calcitriol ENhancing Taxotere]) to determine whether the prostate-specific antigen response rate (defined as a >50% decline in prostate-specific antigen for >1 month) was different for the standard therapy for ADT-resistant PCa (docetaxel 36 mg/m<sup>2</sup> weekly intravenously for 4 weeks every 6 weeks) compared with the same dose and schedule of docetaxel plus DN-101, 45 mg weekly. Although this study did not meet the prostate-specific antigen response criteria, it did alter the overall survival and therefore justified a large randomized trial to assess survival. This trial (ASCENT II) was halted before full recruitment because survival in the DN-101 arm was reduced compared with standard of care [212]. In post-trial analyses, there were modifications to docetaxel regime between ASCENT I and II that could have impacted efficacy, and there is a reasonable argument that this clinical failure has impeded subsequent development of vitamin D-centered therapies for illegitimate reasons [213].

Set against these design challenges, and perhaps nuanced biological responses, it is worth reiterating that the links between VDR signaling and anticancer and antiinflammatory actions appear strongest in AA PCa patients compared with EA counterparts [198]. For example, vitamin D<sub>3</sub> supplementation prior to radical prostatectomy significantly altered tumor expression of genes associated with inflammation in AA compared with EA patients [214]. More recently, independently of genomic ancestry, VDR signaling has been associated with regulation of the circadian rhythm [215], reflecting a wider function for nuclear receptors to regulate this key process (reviewed in Ref. [216]).

Together, these data suggest that AA men, compared with EA counterparts, are more acutely sensitive to low serum vitamin D<sub>3</sub> levels that lead to inadequate VDR signaling; this has potentially been overlooked in the clinical evaluation of vitamin D<sub>3</sub> analogs in PCa cohorts that largely consisted of EA men [211,213].

While the mechanistic association between circadian rhythm and innate immunity in patients with high genomic African Ancestry has not been completely studied, circadian rhythmicity is indeed a central feature of both innate and adaptive immunity [217]. Specifically, circadian rhythmicity has been studied in monocytes and macrophages, which are a critical immune cell type within the innate immune system. This can be extremely influential in AA men with PCa that have persistent inflammation and an immunosuppressive tumor microenvironment. For example, a cohort of 3000 Ghanaian, AA, and EA men with PCa men has been used recently to reveal that African genomic ancestry significantly drives a unique systemic immune-oncological signature [218]. Importantly, the suppression of tumor immunity protein signature associates with metastatic and lethal PCa.

Given that multiple clinical studies have confirmed that the VDR plays a crucial role in modulating innate immune responses toward various pathogens [219–223], similarly epidemiological studies suggest an inverse association between circulating levels of 25(OH)D<sub>3</sub> and inflammatory markers, including CRP and interleukin (IL)-6 [224,225], and elevated IL6 in Ghanaian and AA men, which is well documented, is secreted by disease-associated macrophages [218,226,227].

1,25(OH)<sub>2</sub>D<sub>3</sub> or its analogs have been shown to initiate the differentiation of myeloid progenitors into macrophages, and to reduce MCP-1 and IL-6 expression in macrophages [228] as well as interferon (IFN)- $\gamma$  activation within macrophages [229,230]. Indeed, VDR/RXR activation is enriched in tumor-associated macrophages (TAMs) compared to macrophages from normal tissues [231]. Since higher BMI has been associated as a contributing factor to aggressive PCa in AA men [232], Masseti et al. (2022) observed that macrophages isolated from murine prostate tumors that are PTEN<sup>PC</sup> -/-; SMAD<sup>PC</sup> -/- knockout and treated with high-fat diet compared with a low-fat diet demonstrated increased VDR/RXR activation and tumor cell growth [231]. In further support of ancestral for macrophage activation, health donor macrophages isolated from subjects of African ancestry compared with European ancestry demonstrate a differential response when challenged to bacterial. The authors concluded these findings are ancestry related; as macrophages are one of the most conserved cell populations throughout evolution [233]. This is relevant to patients with 25(OH)D<sub>3</sub> deficiency

as it is required for a defense against the intracellular pathogen *Mycobacterium tuberculosis* [222]. Thus, 25(OH)D<sub>3</sub> deficiency in populations with African ancestry could be responsible for these observations as the signaling of the VDR is more potent in the normal prostate to control inflammatory signals and is perhaps most critically impacted by elevated BMI.

Thus, there appears to be a dual effect of 25(OH)D<sub>3</sub>/VDR to direct activity on the cancers from men of African ancestry. Indeed, in the AA derived RC-43T PCa cell lines express higher levels chemokines as well as an overall elevated interferon immune signature compared with LNCaP cells, and this reversed vitamin D treatment [168]. Similarly, Haridman et al. demonstrated that AA prostate cancer patients treated daily for 4 weeks with 4000 IU of 25(OH)D<sub>3</sub> day prior to prostatectomy demonstrated increased inflammatory suppressive immunity compared with EA patients [14]. While the current data available are limited on the direct role of African ancestry on the immune-circadian rhythm signature in macrophages, it is tempting to speculate that paracrine/autocrine signaling between tumor cells recruits and polarizes macrophages toward a suppressive phenotype. This is highly plausible as macrophage inhibitor treatment of the C4B2 xenograft model, which lacks T cells but has an abundance of macrophages, results in decreased tumor growth [234]. Lastly, since similar findings have been observed for VDR activation in dendritic cells, it is imperative that AA PCa patients be oversampled in larger 25(OH)D<sub>3</sub> clinical trials and that VDR activity in both tumor and immune cell populations will be interesting to explore, for example, at the single cell level.

## 6. Conclusion

The connections between the gene regulation functions of the VDR and the biology of the prostate have been the focus of significant study, and it is clear that there are strong relationships in terms of the biological impact on prostate function, and how this signaling appears to be distorted in PCa. The rationale to define these relationships and mechanisms of corruption is that they would offer a route to exploiting this type II nuclear receptor in a chemoprevention or chemotherapy strategy.

Set against this optimism is that several fundamental biological questions remain, and the precise context to target the VDR in PCa remains ill-defined. It remains unclear how separate or integrated are the functions of the VDR in the absence and presence of ligand. What transcriptional pathways are regulated in either context and to what level are these processes integrated? Somewhat implicit in this uncertainty is that the VDR functions do not stand alone, but rather are integrated with

other nuclear receptors and transcription factors. However, it remains to be resolved as to how this cross-talk occurs be it either adjacent but independent genomic binding or shared protein–protein interactions in a genomic context, and to what extent ligand exposure shifts the distribution, and genomic location, between these functions. Presumably, VDR-AR cross-talk in the prostate and PCa reflects this, but to date there is no clear answer about the importance, extent, and biological function of this cross-talk. Given that the VDR is implicated in the interaction with many different transcription factors, these key questions are amplified further.

It is also becoming clear that genomic ancestry is a major determinant of the VDR actions and interactions. That is, the epidemiological and clinical analyses of VDR signaling appear to have the greatest magnitude of biological impact in men of African genomic ancestry. However, this still remains poorly established due to the historical underrepresentation of AA men in PCa prevention or therapy trials. This is a rapidly changing aspect of PCa, and it is likely that more clear answers will emerge. It is possible that within the context of African genomic ancestry that the VDR signaling lies in a more prominent biological fulcrum, combining biopsychosocial process with gene regulatory scenarios required for prostate health, which are a significant target in the etiology of AA PCa.

## 7. Summary points

- Epidemiologic studies show protective of U-shaped associations between 25(OH)D status and risk of aggressive or lethal prostate cancer.
- Men of African Ancestry have a dual disparity in risk of aggressive prostate cancer and 25(OH)D deficiency.
- In vitro and in vivo studies support anticancer effects of 1,25(OH)<sub>2</sub>D<sub>3</sub>.
- Cistromic approaches have identified key targets and pathways regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub>.

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## Vitamin D and pancreatic cancer

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### OBJECTIVES

- The following objectives are addressed and placed in the context of current knowledge on the etiology, clinical presentation, and biology of pancreatic cancer.
- To update current evidence on the association between vitamin D and pancreatic cancer risk.
- To provide an updated review of the experimental evidence on the effects of vitamin D on pancreatic cancer cells and the associated tumor microenvironment.
- To update current evidence on the association between vitamin D and prognosis of patients with pancreatic cancer.
- To summarize the results of vitamin D intervention studies in patients with pancreatic cancer.

deaths from PC was almost the same as the number of new cases (466,003 and 495,773, respectively) (<https://gco.iarc.fr/today/home>). Incidence and mortality rates are about 3-4fold higher in high, compared with low/medium, human development index countries and are slightly higher in men than in women [1].

Because of changes in age distribution, population growth, the increased prevalence of risk factors, and improvements in PC diagnosis and registration, the incidence rate of PC has increased worldwide in the past years, and it is expected to increase further in the next two decades. Worrisomely, it is estimated PC will become the second cause of cancer-related deaths in the United States by 2030, unless there is significant medical progress [2].

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of PC (>90%) [3], and the latter term will be used for convenience in this review. PDAC has a dismal prognosis, with a 5-year survival rate of <10% [1] and a 10-year survival rate of 1% [4].

### 1. Pancreatic cancer: an overview

Pancreatic cancer (PC) is the 12th most common malignancy worldwide and the 7th leading cause of cancer mortality. Among gastrointestinal cancers, PC ranks fourth in terms of incidence. In 2020, the number of

#### 1.1 Etiology of pancreatic cancer

From an etiological perspective, PC is a complex disease with several risk factors having been identified, each with relatively modest contributions. Identifying

a Co-first authors.

b Co-senior authors.



the causes of PC is a must to (1) implement primary prevention interventions and (2) define high-risk individuals who are candidates for screening.

The main nongenetic, nonmodifiable, risk factors for PC are older age (most cases occur after the age of 65 years) and ethnicity (in the United States, risk for black populations is  $\geq 1.5$ -fold higher than that for white populations). The leading modifiable risk factor for PC is cigarette smoking. A metaanalysis on the association of smoking with the risk of PC found an odds ratio of  $\sim 1.7$  for current smokers compared with never-smokers [5]; a more recent and comprehensive assessment confirmed this risk magnitude [6]. Smoking cessation reduces risk and, by 14–20 years after cessation, the risk for former smokers drops to that of never-smokers [7,8]. A summary of metaanalyses indicates that the population attributable risk (PAR) for smoking ranges across studies and global regions from 11% to 32% [9]. Heavy alcohol consumers are at 1.6-fold increased risk, compared with abstainers and occasional drinkers [10].

Obese individuals have  $\sim 1.6$ -fold increased risk in comparison with those with normal weight [11]. Type II diabetes (T2D) is also a well-established risk factor. Individuals with T2D have 2.5 times higher PC risk, the risk being higher for new onset T2D (OR  $\sim 6$ ) and insulin users (OR = 3.7) [12]. The relationship between obesity and PC is complex, and results from this study suggest a noncausal relationship between long-standing T2D and obesity and PC risk. On the contrary, PC seems to be causal of new-onset diabetes.

Chronic pancreatitis (CP) is also associated with increased PC risk. For individuals with sporadic CP, the increase in risk may be in the range of 2–5-fold [13,14]. However, the risk is much higher (in the range of 60-fold) for subjects with hereditary CP [15].

Epidemiological studies have also identified a few factors that are associated with a reduced risk of PC. Among them are asthma and allergy, which are associated with a ca. 40% risk reduction [16–18]. Physical activity and dietary intake of fruits and foods containing folate may be associated with a lower PC risk; however, the evidence is unclear [19,20].

ABO blood type has been reported to be associated with PC risk [21,22]. Compared with participants with blood group O, those with A, AB, or B blood groups were at higher risk of developing PC (OR = 1.32, 1.51, and 1.72, respectively) [22].

The oral and gut microbiome have received great attention in the past years. *Porphyromonas gingivalis* has been proposed to be involved in PC development [23,24]. Kartal et al. [25] identified a fecal microbiota signature that can predict PDAC with high sensitivity and specificity. However, whether the association is causal needs to be formally established.

Familial aggregation of PC occurs in approximately 5%–10% of cases [26] as a consequence of inherited genetic changes, shared environmental factors, or the joint actions of both [27]. Family history of PC in first-degree relatives has been consistently associated with increased PC risk [18,28–31]. Results from observational studies suggest that having at least one relative with PC increases the risk of PC between 1.5- and 13-fold [27].

## 1.2 Genetic susceptibility

Germline genetic risk factors may be classified as mutations or common variants on the basis of their frequency: mutations contributing to hereditary syndromes and variants to sporadic cases [26]. Approximately 2% of all cases correspond to hereditary syndromes (hereditary PC, HPC) caused by low-frequency, highly penetrance, germline mutations in *BRCA2* or *BRCA1* (hereditary breast-ovarian syndrome), *p16/CKDN2A* (familial atypical multiple mole melanoma syndrome), *PRSS1*, *PRSS2*, *SPINK1*, or *CTRC* (hereditary chronic pancreatitis), *STK11/LKB1* (Peutz-Jeghers syndrome), *CFTR* (cystic fibrosis), *APC* (familial adenomatous polyposis), *TP53* (Li-Fraumeni syndrome), *MLH1* or *MSH2* (Lynch syndrome), and *ATM* (DNA repair) [18,26,27].

Approximately 90% of PC cases are sporadic. A total of 16 regions harboring common single-nucleotide polymorphisms (SNPs) with modest risk effects have been identified through genome-wide association studies (GWAS) in populations of European ancestry [18,32–36]. A recent PC GWAS complemented with genome spatial autocorrelation analysis and functional in silico analysis of public genomic information and Hi-C interactions identified 51 additional candidate variants in 17 genetic susceptibility regions associated with PC risk [37].

Despite the effort placed in identifying PC risk factors, there is a great need to emphasize prevention. The low prevalence of PC hampers the use of preventative/early diagnosis/screening strategies in the general population, and the identification of subjects with a higher risk of PC remains a major goal. This is the main scenario where a putative role of vitamin D might play a role in the future.

## 1.3 Clinical presentation and treatment

PC is one of the most challenging tumors to treat: most patients present with locally advanced or metastatic disease, and up to 30% of patients have cachexia at the time of diagnosis. Together with the advanced age, these factors impose significant therapeutic limitations. Radical surgery can only be considered in

approximately 20% of patients and should be carried out only in high-volume centers. Several clinical trials have shown the benefit of adjuvant chemotherapy [38–40]. Recent advances in the use of neoadjuvant chemotherapy support its benefit in patients with borderline resectable disease [41,42]. In patients with metastatic disease, the two main therapeutic schemes are FOLFIRINOX (or variants thereof) and gemcitabine/abraxane; the former has superior antitumor effects but also has a greater toxicity and is generally reserved for patients with better performance status. Nevertheless, the overall life expectancy of patients with metastatic PC is 6 months. The role of radiotherapy in the management of PC is controversial, particularly in the perioperative setting. PC has not yet benefitted from the impact of immune therapies in the standard setting. PC patients often need to receive supportive care to mitigate weight loss, including digestive enzyme replacement due to exocrine pancreatic insufficiency. PC is also associated with pain, sometimes excruciating, that requires analgesics, including opioids.

Undoubtedly, improved therapies are necessary to reduce PC-related mortality, but significant progress will require advances in multiple research areas.

## 2. Pancreatic cancer and vitamin D: epidemiological studies

The relationship between sun exposure, vitamin D, and PC risk has been studied in several types of epidemiological studies, including ecological, case–control, and cohort studies. Overall, these studies have provided inconsistent results. The strategy used to review the available information is shown in Fig. 92.1. The main findings are summarized in Tables 92.1–92.3 (Box 92.1).

### 2.1 Solar Ultraviolet-B radiation

Both ecological and observational studies have reported the existence of a gradient, with inverse relations between solar UVB radiation exposure—using metrics such as latitude, solar radiation data, temperature, time spent outdoors, or other UV radiation-related factors—and the incidence and/or mortality rates due to PC.

*Ecological studies* have leveraged on various types of data (e.g., geographical, solar radiation, or temperature) to investigate the association of solar UVB dose or exposure with PC incidence and/or mortality rate using country-specific or multicountry data.

*Geographical* ecological studies, for example, those carried out in Japan [43,44] and Spain [45,91], among others, have consistently found an inverse relationship between latitude and PC incidence/mortality rates.

*Ecological studies* using solar radiation data in specific geographical regions (i.e., countries) have also reported an inverse relationship. Mizoue [47] used annual hours of solar radiation received from 1961 to 1990 as measurement in Japan and observed a moderate, inverse, association with six gastrointestinal cancers, including PC. A similar inverse relationship with PC risk was found by Kinoshita et al. [48] using the average of daily-accumulated solar radiation (1971–2000) as measurement, using data from the Japan Meteorological Agency. They also reported that low temperature might associate with an increased PC risk. In the United States, Grant [49] found an inverse association between measurements of UVB radiation and PC mortality rates. Similar findings were reported by Boscoe and Schymura [50]. Mohr et al. [46] found that, with occasional exceptions, countries from both hemispheres with lower UVB irradiance had higher age-standardized incidence rates of PC for both sexes (International Agency for Research

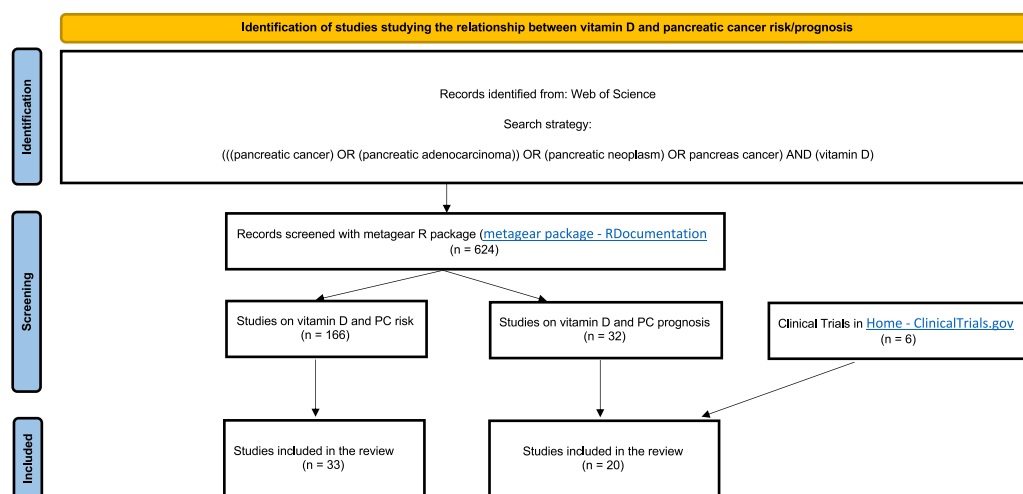


FIGURE 92.1 Summary of the effects of vitamin D on pancreatic cancer cells and the tumor microenvironment.

**TABLE 92.1** Summary of the epidemiological studies on the association between vitamin D and pancreatic cancer.

Vitamin D related measurement	References	Study design	Population characteristics	Association
Solar ultraviolet B radiation				
Latitude	[43–45]	Geographical ecological studies	Japan, Spain	Inverse relationship with PC incidence/mortality
Solar radiation data	[46–54]	Ecological studies	Japan; United States, Multicountry	Inverse relationship
	[55]	Case–control study	Queensland PC study	Inverse relationship
	[56]	Prospective study	NIH-AARP Diet and Health study	Nonlinear relationship
	[36]	Metaanalysis		
Low temperature	[48]	Ecological studies	Japan	Inverse relationship with mortality rates
Ethnicity	[55]	Case–control study	Queensland PC study	Inverse relationship
Circulating serum levels of 25(OH)D	[46]	Ecological study	28 regions in 18 countries	Sigmoid inverse dose response
	[57]	Cohort study	ATBC cohort (Male Finnish smokers)	Direct association
	[58]	Cohort study	PLCO study	No association
	[59]	Pooled analysis of eight cohorts (VDPP)	ATBC, CPS-II, MEC, NIST, NYU-WHS, PLCO, SMHS, SWHS	Direct association with a high 25(OH)D concentration ( $\geq 100$ nmol/L) No associations with lower levels No association with prediagnostic status
	[60]	Nested case–control study	PLCO	Direct association with high 25(OH)D levels ( $\geq 100$ nmol/L) No associations with lower levels
	[36]	Cohort study	HPFS, NHS, PHS, WHI and WHS	Inverse relationship

Circulating VDBP	[61]	Nested case–control study	EPIC (626 cases/626 controls) HUNT2 (112 cases/112 controls)	No association
	[62]	MR analysis using five 25(OH)D genetic markers (GC-rs2282679-G, <i>CYP2R1</i> -rs10741657-G, <i>CYP2R1</i> -rs116970203-A, <i>DHCR7</i> -rs12785878-G, and <i>CYP24A1</i> -rs6013897-A)	UKBiobank UKBiobank + PanScan	No association
	[63]	Nested case–control study	ATBC study	No association
	[60]	Nested case–control study	PLCO	No association
Oral intake of vitamin D				
Dietary intake based on quantitative food-frequency questionnaires	[64]	Prospective study	Two prospective studies: HPFS and NHS	Inverse
	[65]	Case–control	Italy (326 cases and 652 controls)	No association
	[66]	Case–control	USA (532 cases and 1701 controls))	In men, direct association No association in women
	[67]	Metaanalysis	14 prospective cohorts: ATBC, BCDDP, CNBSS, CPS II, CTS, COSM, HPFS, IWHS, MCCS, NLCS, NYSC, NHS, PLCO, SMC	No association
	[68]	Pooled analysis of nine PanC4 case–control studies	(“NHANES III (1988–1994),” n.d.) NHANES III.	Direct association

Continued



**TABLE 92.1** Summary of the epidemiological studies on the association between vitamin D and pancreatic cancer.—cont'd

Vitamin D related measurement	References	Study design	Population characteristics	Association
			Hyattsville, MD: Centers for Disease Control and Prevention (1998)[207] [208] [209] [66] [55][210] [211]	
	[69]	Metaanalysis of observational studies	[65] [70] [58] [64] [71] [66][212]	No association Inverse association after considering only the prospective studies and the dose–response was evaluated
Intake of dairy products	[67]	Meta-analysis of 14 cohort studies	14 prospective cohorts: ATBC, BCDDP, CNBSS, CPS II, CTS, COSM, HPFS, IWHS, MCCS, NLCS, NYSC, NHS, PLCO, SMC	No association
Supplementary vitamin D intake	[68]	Pooled analysis of nine PanC4 case–control studies	[207] ("NHANES III (1988–1994)," n.d.)NHANES III. Hyattsville, MD: Centers for Disease Control and Prevention (1998)[208] [209] [66] [55][210] [211]	Direct association
Vitamin D index based on both oral intake and vitamin D production	[72]	Prospective study	[65] HPFS	Inverse relationship
Vitamin D status	[73]	Metaanalysis	9 studies: [57] [64] [58]	No association

VDR-rs2228570-C:  
direct association  
VDR-rs1544410-A:  
inverse association

**TABLE 92.2** Summary of the studies reporting on the association between vitamin D and pancreatic cancer prognosis.

Vitamin D related measurement	References	Study design	Population characteristics	Association with overall survival
<i>Solar ultraviolet B radiation</i>				
Latitude	Grant et al. [45]	Ecological study	579,000 males and 550,000 females from Eurocare-3	
<i>Circulating serum levels of 25(OH)D</i>				
	Cho et al. [78]	Retrospective study	114 PC patients with advanced disease from Siteman Cancer Center	Direct association
	Yuan et al. [79]	Prospective study		Direct association
	Weinstein et al. [80]	Prospective study	143 PC cases from the ATBC study, composed of Finnish male smokers, aged 50–69 years, were recruited from 1985 to 1988 to participate in a controlled primary prevention trial	Direct suggestive association
	Van Loon et al. [81]	Prospective study	256 advanced PC cases	No association
	Haas et al. [82]	Prospective study	59 PC cases (locally advanced or metastatic disease) undergoing palliative first-line chemotherapy	No association
	McGovern et al. [83]	Retrospective study	627 PC cases	No association
	Von Hoff et al. [84]	Post hoc exploratory analysis	422 metastatic PC cases of the phase III MPACT trial	No association
<i>Genetics</i>				
SNPs tagging VDR gene				
36 tagging SNPs	Yuan et al. [79]	Prospective study	493 PC cases from five US cohorts	No association
VDR-rs2853564	Innocenti et al. [85]	Prospective study	294 advanced PC cases treated with gemcitabine (CALGB 80303) and 408 PC cases treated with gemcitabine of no chemotherapy (Mayo Clinic)	VDR-rs2853564-G with better survival VDR-rs2853564 interacting with gemcitabine treatment (G allele + gemcitabine had better survival) VDR-rs2853564 interacting with pretreatment levels of 25- 25(OH) D (G allele + high levels had better survival)
<i>Molecular features</i>				
Expression of VDR	Wang et al. [86]	Retrospective series	61 PC patients from First Hospital of China Medical University	Direct association (low levels → poorer survival)
VTDB levels	Iuga et al. [87]	Prospective series	9 patients from the Regional Institute of gastroenterology and Hepatology<	Direct association (low expression level → poorer survival)
CYP24A1 expression	Gao et al. [88]	Prospective series	73 surgical PDAC cases	No significant association

**TABLE 92.3** Summary of the vitamin D intervention studies in patients with pancreatic cancer.

<b>Intervention</b>				
Seocalcitol	Evans et al. [89]	Phase II clinical trial	43 cases with inoperable PC	No significant association
Docetaxel + calcitriol versus docetaxel	Blanke et al. [90]	Phase II clinical trial	25 patients with unresectable locally advanced/metastatic pancreatic adenocarcinoma	No significant association
High-dose Vitamin D <sub>3</sub> versus standard-dose <sup>a</sup>	NCT03472833	Randomized phase 3 clinical trial	25 patients with vitamin D deficiency	Terminated (slow recruitment, patients lost to follow-up due to Covid-19 pandemic) Prognosis was not included as outcome-no results posted
Pembrolizumab + paricalcitol versus Pembrolizumab + placebo	NCT03331562	Double-blind randomized placebo-controlled phase II clinical trial	24 patients	Completed No difference
Gemcitabine + nab-paclitaxel + paricalcitol versus gemcitabine + nab-paclitaxel + placebo	NCT03520790	Randomized phase I/II clinical trial	112 patients with stage IV pancreatic cancer	Recruiting
Single Group assignment of advanced metastatic pancreatic cancer patients Paricalcitol in Combination with gemcitabine/nab-paclitaxel	NCT04617067	Phase 2 clinical trial	15 participants	Active, not recruiting
Arm A: 50 mcg paricalcitol IV weekly Arm B: 12 mcg paricalcitol PO daily	NCT03300921	Phase Ib nonrandomized Study	3 participants with resectable PC	Terminated (newer research suggested that paricalcitol may be harmful to a subset of pancreatic cancer patients, and it is not feasible to subtype patients in the short time frame before surgery)
Nivolumab/gemcitabine/nab-paclitaxel + paricalcitol versus nivolumab/gemcitabine/nab-paclitaxel	NCT03519308	Early phase 1 clinical trial	9 patients with resectable PC	Terminated (the accrual goal could not be met and the drug manufacturer pulled support)
Arm A: 5-FU + leucovorin + liposomal irinotecan + Paricalcitol 75 mcg Arm B: 5-FU + leucovorin + liposomal irinotecan + Paricalcitol 7 mcg/kg	NCT03883919	Phase 1 nonrandomized	20 patients with advanced PC progressed on gemcitabine-based therapy	Active, not recruiting
Single Group assignment gemcitabine, abraxane,	NCT02336087	Pilot phase 1	21 patients with unresectable PC	Active, not recruiting

Continued



**TABLE 92.3** Summary of the vitamin D intervention studies in patients with pancreatic cancer.—cont'd

Intervention				
metformin, Dietary Supplement including vitamin D				
Paricalcitol, hydroxychloroquine, chemotherapy	NCT04524702	Phase 2 single group	21 patients with advanced or metastatic PC	Recruiting
Paricalcitol + abraxane + Gemcitabine versus abraxane + Gemcitabine	NCT02030860	Randomized pilot/ pharmacodynamic/Study	15 patients with resectable	Completed. No results posted
Paricalcitol + cisplatin + abraxane + Gemcitabine	NCT03415854	A Phase II Pilot trial with single group assignment	14 patients with previously untreated metastatic PC	Active, not recruiting

<sup>a</sup>High dose. Patients will receive a high dose—180,000 I.U. (1 drop equals 400 I.U.) of vitamin D<sub>3</sub> orally on day 1, and then 4000 I.U. for 60 days; Standard dose. Patients will receive a standard dose—800 I.U. (equals two drops) of vitamin D<sub>3</sub> orally for 60 days.

**BOX 92.1****Summary of the main epidemiological findings**

- Epidemiological studies exploring the relationship between vitamin D and PC risk, using various designs and exposure assessment strategies, have provided inconsistent results.
- Mendelian randomization analyses do not support a causal link between vitamin D levels and PC risk.
- Intervention *studies* have not assessed the impact of vitamin D supplementation on the incidence of PC.
- Clinical trials testing the effect of the administration of vitamin D analogs in combination with chemotherapy did not improve overall survival.

on Cancer [IARC] Global Cancer [GLOBOCAN] database [latest year for which complete data were available was 2002]).

In a review of ecological studies from multiple countries, Grant [51] reported a consistent strong inverse correlation between *solar UVB* exposure and risk for 15 types of cancer, including PC. Similar results were obtained by Moukayed and Grant [52] in a later review. Garland et al. [53] analyzed the association of UVB irradiance measured using NASA satellite data, adjusted for cloud cover, and the age-standardized PC incidence rates, obtained from GLOBOCAN in 2008. They found that both male and female residents of countries with low UVB irradiance had approximately sixfold higher PC incidence rates than those of countries with high UVB irradiance, after adjustment for traditional PC risk factors.

Regarding sex, the reports have been discordant. Mizoue [47] found that the inverse correlation was stronger for men ( $\rho = -0.51; -0.53$  vs.  $\rho = -0.32; -0.31$ ), and Boscoe and Schymura [50] showed evidence for a north–south gradient PC incidence/mortality gradient only among females. In a review, Grant [51] reported that the inverse correlation was significant for males ( $\rho = -0.46$ ,  $P$ -value = 0.005) and borderline for females.

Although these ecological studies are useful for hypothesis generation, they have important limitations, including the use of grouped data: the impact of climatic factors on the human body varies depending on individual lifestyles and occupations, UVR exposure may be a proxy or marker of some other cancer risk factor, and it may be difficult to appropriately control for confounders, among others. In the case of PC, a specific confounder that needs to be considered is depression since it may be an early symptom of PC [92], and it is also inversely associated with the intensity of solar radiation [93]. Another possible confounder is the consumption of animal products. For example, Grant et al. [54] used age-adjusted incidence rates for 21 cancers for 157 countries in 2008, considering dietary supply among other

confounding factors, and found that the correlation between PC incidence and latitude decreases when the animal product consumption was considered.

Other epidemiological designs providing a higher level of evidence than ecological studies have been used to assess the association between UVR exposure and PC risk, including observational case–control, cohort studies, and metaanalyses. In a case–control study conducted in Australia, Tran [55] analyzed the association between UVR exposure—using data collected through questionnaires and NASA’s Total Ozone Mapping Spectrometer to estimate *ambient UVR*—and PC risk. They showed that being born in or living in areas with higher ambient UVR was associated with ca. 30%–40% lower risk of PC. Prospective studies have also confirmed this association: Lin [56] examined ambient UVR exposure and PC risk in the National Institutes of Health (NIH)-AARP Diet and Health Study. After adjusting for multiple potential confounders (e.g., gender, age, BMI, diet, smoking, education, and physical activity), they found a nonlinear relationship, suggesting that different biological mechanisms may underlie the association.

## 2.2 Oral intake of vitamin D

Approximately 10% of the endogenous vitamin D comes from the diet—cholecalciferol (vitamin D<sub>3</sub>)—with egg yolk, liver, or oily saltwater fish being the main animal sources. The plant-derived ergocalciferol (vitamin D<sub>2</sub>) is found in mushrooms, among others. Food fortification is an additional dietary source.

The lack of reliable biomarkers of long-term exposure to specific dietary components has led to the use of recall methods, such as quantitative food-frequency questionnaires, to assess the association with many cancers, including PC. The results of such studies have been contradictory, probably due to confounding as well as to the complex association between intake of dietary vitamin D, calcium, or other vitamins (e.g., retinol) and PC

risk. Some studies have found that vitamin D intake is protective [64,67,69], whereas others have not found an association [65,67,69] or have even made opposite observations [66,68,69].

*No significant association.* In a case–control study from Italy, Bravi et al. [65] studied micronutrients and PC risk (vitamin D intake being categorized in quintiles), reporting a nonsignificant positive association. In a pooled analysis of 14 cohort studies from Europe, North America, and Oceania including subjects of a diverse age range (15–107 years), no statistically significant associations were observed between dietary vitamin D during adulthood and PC risk [67]. In a metaanalysis of observational studies comparing high dose versus low dose, no significant difference in PC risk was reported [69].

*Protective factor.* Skinner et al. [64] found, in two large prospective cohort studies (HPFS and NHS), that intake of 300–449 IU vitamin D<sub>3</sub>—ascertained before PC detection using food frequency questionnaires—was associated with a 43% decreased incidence of PC. They also found that, compared with participants consuming <150 IU/d of vitamin D, those who consumed >600 IU/d had 41% lower risk of PC after adjusting for retinol intake. In these studies, they did not find a significant association with either total calcium intake or calcium from food after adjusting for total vitamin D intake. They also investigated the association with the dietary items contributing vitamin D and nonsignificant associations with PC risk were reported. Giovannucci et al. [72] found decreased relative risks for PC, among other cancers, among subjects with a high vitamin D status index, derived from multiple factors including oral intake, among men enrolled in the Health Professionals Follow-Up Study. A recent metaanalysis of three prospective studies [64,70,71] reported a decrease of 25% of PC risk for a vitamin D (10 µg) intake [69].

*Risk factor.* In a population-based case–control study, using a semiquantitative food-frequency questionnaire, Zablotska et al. [66] reported different risk patterns among men and women. In men, an increased PC risk was associated with currently recommended dietary vitamin D intake levels (highest [ $\geq 450$  IU/day] versus lowest [ $< 150$  IU/day] intake, OR = 2.6, trend- $p = .009$ ) and total vitamin D intake from diet and supplements (for  $< 800$  IU/day), even after adjusting for potential confounders such as calcium and retinol intake. However, no associations were found in women.

In a pooled analysis of 2963 PC cases and 8527 controls from the Pancreatic Cancer Case–Control Consortium (PanC4), a modest significant positive association was found between dietary vitamin D intake and PC risk (OR = 1.13 per 100 IU/day; OR  $\geq 230$  vs.  $< 110$  IU/day = 1.31) [68]. This association seemed to be stronger in people with low retinol/vitamin A

intake. The same study investigated the role of supplementary vitamin D intake in relation to PC risk and found no association. However, when the estimates of one of the studies (the Queensland Pancreatic Cancer Study) were excluded, the pooled OR for supplementary intake ( $\geq 400$  IU/day vs. none) increased from 1.03 to 1.20 (95% CI 0.95–1.50,  $P$ -heterogeneity = 0.35) and the pooled OR for total intake became significant (OR = 2.01, 95% CI 1.50–2.69,  $P$ -heterogeneity = 0.66).

The relationship between vitamin D intake and PC risk has been also investigated using the consumption of dairy products as proxy, since they naturally contain calcium and are often fortified with vitamin D. Unlike in other gastrointestinal cancers, no associations of dietary calcium and vitamin D with PC risk were found [94]. In an analysis of 14 cohort studies, no association between PC risk and intake of dairy products—including total milk, whole milk, low fat milk, cheese, cottage cheese, ice cream, or yogurt—was found [67].

One important caveat of the studies described before is that the effects of vitamin D may be counteracted/modified by a wide range of factors, which may explain the inconsistent findings. Zablotska et al. [66] found no evidence of statistically significant interactions between vitamin D intake and retinol in relation to PC risk. These authors found weak evidence that smoking is a modifier of the effect of vitamin D intake on PC risk in women, but not in men. Genkinger et al. [67] found that smoking significantly modified the associations between cheese and total vitamin D and PC risk, so that former smokers who consumed cheese had an increased risk, while this association was not significant in non- or current smokers.

### 2.3 Ethnicity

The differences in PC incidence and mortality according to ethnicity have led to investigate this variable, since vitamin D insufficiency is more common in the black population compared with others [95]. Several studies have found a high prevalence of vitamin D insufficiency in dark-skinned people [72], likely resulting from higher amounts of melanin, which effectively filters UV-B radiation, with possible contributions from other lifestyle differences. In a study of male health professionals, Giovannucci et al. [72] found a higher cancer mortality (and to a lesser extent, in incidence) among blacks than in their white counterparts, despite comparable socioeconomic status, medical knowledge or use of screening tests, or adherence to a healthy lifestyle and dietary habits. In an Australian case–control study, Tran [55] found that people with fair skin had 47% (95% CI 0.37–0.75) lower risk of PC than those with dark skin. However, a causal relationship has not been established.

## 2.4 Genetics

Several epidemiological studies have explored the role of polymorphisms in *VDR* and genes in the vitamin D pathway in relation to the risk of multiple cancers, including PC. However, there is no evidence for a substantial influence of any single common variant on PC risk.

In a population-based case–control study investigating the association between 87 SNPs in 11 vitamin D pathway genes and PC risk, Anderson et al. [75] did not find any significant association. Similar observations were made by Arem et al. [76]. However, in a small case–control study conducted in China, Li et al. [77] found two polymorphisms in *VDR* associated with PC risk (*VDR*-rs2228570-C: direct association; *VDR*-rs1544410-A: inverse association).

Dimitrakopoulou et al. [96] explored a polygenic score for circulating 25-hydroxyvitamin D (25(OH)D, calcidiol) concentrations, using four SNPs (*GC*-rs2282679, *CYP2R1*-rs10741657, *DHCR7*-rs12785878, and *CYP24A1*-rs6013897), and found little evidence of association with the risk of any of seven incident cancers analyzed, including PC.

## 2.5 Serum/plasma levels of vitamin D

Several studies with different epidemiological designs have been conducted to evaluate the association of serum 25(OH)D, reflecting vitamin D status from both sun and diet, with PC risk. However, the results are conflicting.

Ecological studies analyzing the relationship between estimated serum 25(OH)D levels and PC incidence rate have found an inverse dose–response association that follows a sigmoid curve [46]. Three prospective studies obtained information on circulating 25(OH)D levels. In the ATBC cohort of male Finnish smokers, subjects with higher vitamin D levels had nearly threefold increase in PC risk (highest vs. lowest quintile: OR = 2.92, 95%CI: 1.56–5.48, *p*-trend = 0.001) [57]. However, these results may not be generalizable to other populations such as nonsmokers, women, or residents from other geographical areas. These results were not replicated overall in the PLCO study, conducted in the United States: among individuals with low UVB residential exposure, higher 25(OH)D concentrations were positively associated with PC risk [58]. The Vitamin D Pooling Project, including eight cohorts (among them, the ATBC and PLCO) [74], aimed to investigate the relation of 25(OH)D and six rare cancers [59]. They validated the previously reported results for PC, showing an increased risk associated with very high levels ( $\geq 100$  nmol/L; OR<sub>adj</sub> = 2.12, 95% CI: 1.23, 3.64) [71]. This association remained in a sensitivity analysis

upon removing each individual study, and it was independent of sex, ethnicity, obesity, and diabetes mellitus. However, no significant associations were observed for participants with lower vitamin D status, and no reduced risk between prediagnostic status and PC risk was found overall. The results suggested a nonsignificant U-shaped association when cases occurred during the first 5 years of follow-up were excluded. However, Baggerly and Garland [97] pointed out that the U-shaped curve could be a statistical artifact due to the cut-off points chosen to define the groups.

Piper et al. [60], analyzing a higher number of cases from the PLCO study, validated the association between very high levels of serum 25(OH)D and PC risk described before. There was no significant association for individuals with very low levels ( $< 25$  nmol/L). Contrarily, Wolpin et al. [36] found, in five large prospective cohorts, that participants with higher plasma levels of 25(OH)D had lower PC risk (*p*-trend = 0.03). The inverse relationship was also found in the subgroup analyses conducted in white women and men.

Unlike the reports described before, others did not find a significant association. Shang-long et al. [73] performed a metaanalysis of nine studies with data on circulating 25-OH-D levels and found no evidence of association (OR = 1.04; 95% CI 0.93–1.17). Similarly, in a combination of European cohort studies (EPIC and HUNT2) including 738 primary incident PC cases and matched controls, van Duijnhoven et al. [61] found that higher prediagnostic vitamin D concentrations were not associated with PC risk. Recently, in a Mendelian randomization study performed using the resources of UK Biobank, it was evaluated whether genetically predicted 25(OH)D concentrations are associated with overall cancer susceptibility and cancer mortality (including 500 PC cases) using five 25(OH)D genetic markers (*GC*-rs2282679-G, *CYP2R1*-rs10741657-G, *CYP2R1*-rs116970203-A, *DHCR7*-rs12785878-G, and *CYP24A1*-rs6013897-A) [62]. Results ruled out all but very small effects, suggesting that an overall increase in 25(OH)D levels does not reduce the overall cancer risk. As for PC, MR estimates for the association of a 20 nmol/L increase in 25(OH)D concentration were nonsignificant using either the resources of UK Biobank only (OR = 1.09; 0.63–1.88) or in combination with the PanScan study (OR = 1.21; 0.87–1.68). These results do not support the implementation of vitamin D supplements in the general population to reduce PC risk, although individuals with extreme deficiency might benefit.

Other aspects have also been analyzed, and no evidence of significant interactions between the 25(OH)D status and PC risk factors or exposures have been identified, including month at blood draw [36], region of residence [36], age [36], smoking status [36,57,58,61], sex [58,61], physical activity [36,58,61], BMI [36,61], alcohol [61],



diabetes [61], fasting time [36], total vitamin A intake [57,58,61], calcium [61], or multivitamin use [36,61].

Of note, Stolzenberg-Solomon et al. [58] found a significant interaction between annual solar UVB exposure and 25(OH)D status in relation to PC risk: individuals with low estimated annual UVB residential exposure with high 25(OH)D concentrations had a higher PC risk than those with low 25(OH)D concentrations. However, among subjects with moderate to high residential UVB exposure, 25(OH)D concentrations were not associated with PC risk.

### **2.5.1 Vitamin D insufficiency/deficiency in patients with PC**

Vitamin D insufficiency ( $\geq 20$  and  $< 30$  ng/mL serum 25(OH)D) and deficiency ( $< 20$  ng/mL serum 25(OH)D) are common among patients with PC; it is not clear whether this results from insufficient vitamin D intake or insufficient endogenous 25(OH)D synthesis in the liver. The effects derived from pancreatic resection should also be considered.

As part of an intervention study, Klapdor et al. [98] found that 92.4% of 103 PC patients had 25(OH)D levels  $< 30$  ng/mL at the time of patient recruitment. In a retrospective study of 178 PC patients, Cho et al. [78] found that 49% were vitamin D deficient and an additional 25% were vitamin D insufficient. In their series, low vitamin D levels were independent of the season or their BMI. In the context of a randomized clinical trial (CALGB 151006) including 256 patients, Van Loon et al. [81] found that 44.5% and 32.4% of patients were vitamin D deficient or insufficient, respectively. McGovern et al. [83] studied 627 previously untreated PC patients and found that 47.2% and 30.0% were 25(OH)D insufficient or deficient, respectively. Using data from five prospective cohorts, Yuan et al. [79] reported that 33% and 43% of them had deficient or insufficient levels, respectively. A variety of factors may influence the serum levels of vitamin D, including ethnicity, BMI, disease stage, and season of sample collection. Studies have found that the median 25(OH)D level was significantly lower among patients with stage I and II disease and among nonwhite patients [78,81,83], and McGovern et al. [83] found a significant inverse association with BMI, in line with the results reported in healthy individuals. A potential explanation of the lower vitamin D levels found in obese and overweight individuals is that vitamin D accumulates in subcutaneous fat. Longitudinal studies should shed light on this relationship.

### **2.5.2 Predicted levels of plasma 25(OH)D**

Some studies have used predicted levels of plasma 25(OH)D to assess associations, when no direct measurements were available. Obviously, the lack of direct analytical data is a major limitation. Bao et al. [71]

used a score composed of multiple major determinants of vitamin D status (e.g., race, geographic region, vitamin D intake, body mass index (BMI), and leisure time physical activity) to predict long-term plasma 25(OH)D levels [72]. They found that individuals with higher 25(OH)D score were at lower risk of PC. This association held in the analyses stratified by gender. There were no significant interactions with sex, age, follow-up years, region, smoking status, BMI, physical activity, dietary vitamin D intake, retinol, calcium, multivitamin, or supplemental vitamin D use.

### **2.5.3 Circulating vitamin D-binding protein**

The effects of vitamin D are mediated a variety of biological factors in addition to the VDR, including the levels DBP, a member of the albumin, and alpha-fetoprotein gene family that plays a major role in the transport of vitamin D metabolites in blood. Vitamin D is routed to the liver by DBP, where it is converted to 25(OH)D. In a metaanalysis comprising 28 independent studies of a variety of tumors, including PC (two studies [63,75]), Tagliabue et al. [99] found a suggestive inverse link (OR = 0.75; 95% confidence interval [CI], 0.56–1.00) between DBP levels and cancer risk. However, neither Weinstein et al. [63] nor Piper et al. [60] found a significant relationship between DBP levels and PC risk.

Interestingly, the study of Weinstein et al. [63] found a significant interaction between prediagnostic DBP and 25(OH)D levels in relation to PC risk among Finnish men (Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study): the association was inverse in men with high 25(OH)D levels but not in those with low 25(OH)D levels. Moreover, among men with higher 25(OH)D levels, those with low serum DBP showed fivefold elevated risk of PC, while in those with high DBP the association with PC risk was weaker. There were no significant associations between DBP levels and fat intake, vitamin D, calcium, ethanol, smoking, and years between blood collection and study inclusion.

### **2.5.4 Vitamin D status**

A metaanalysis considering vitamin D status and including nine papers (six with data on circulating 25-OH-D levels, two reporting dietary intake of vitamin D, and one examining the effect of previous extensive sun exposure on PC risk) did not find evidence of the association between vitamin D status and PC risk [73].

## **3. The biology and genetics of pancreatic cancer**

### **3.1 The somatic genetics of pancreatic cancer**

Four major genes are altered in PDAC: the oncogene *KRAS* and the tumor suppressor genes (TSG) *CDKN2A*,

*TP53*, and *SMAD4*. Following the approach applied to study colon cancer development/progression and based on the definition of putative preneoplastic lesions found/enriched in the pancreas of patients with PDAC, a progression model has been established [100]. The morphology of PDAC and of the preneoplastic lesions defined as Pancreatic Intraepithelial Neoplasms (PanIN) pointed to a ductal origin, but the cell of origin of human PDAC remains unknown. The accepted progression model suggests that preneoplastic lesions evolve from PanIN-1 (low grade) to PanIN-3 (high grade) stages [101]. Low-grade PanINs are mucinous hyperplastic lesions that can display a flat or papillary morphology, low proliferation, and lack dysplastic features. High-grade PanINs are characterized by less abundant mucus-containing cells, dysplasia, and higher proliferative activity [102]. Other, less frequent, PDAC precursors include intraductal papillary tumors and mucinous cystic tumors. There is essentially no information on the relevance of vitamin D in the latter; therefore, this chapter will focus on the PanIN-PDAC pathway.

The genetic analysis of PDAC was paralleled by the analysis of PanINs, leading to a model that supported that *KRAS* mutations represent the initiating event [103]. Thus, *KRAS* hotspot mutations occur in >90% of PDAC, and they can also be detected in a high fraction of low-grade and high-grade PanINs. Recent evidence suggests that mutant:wild-type allelic imbalances occur during tumor progression [104,105]. The model posits that the inactivation of TSGs drives the evolution toward high-grade PanINs/PDAC. *CDKN2A* is inactivated in almost all PDAC through various mechanisms including point mutations, allelic losses, and epigenetic silencing. *CDKN2A* genetic alterations have been found in PanIN-2—but not in PanIN-1—lesions, and they are thought to precede *TP53* and *SMAD4* alterations. The p53 pathway is inactivated almost universally in PDAC, largely through point mutations but also through allelic losses and MDM2 amplification. *SMAD4* is mainly inactivated through homozygous deletions (in approximately 50% of tumors), but point mutations/deletions occur in an additional 10% of tumors [103]. Genetic alterations in other members of the TGF- $\beta$  receptor signaling pathway also contribute to its inactivation in a subset of PDAC. The frequency of alterations in these genes varies across studies, largely because PDAC is a very desmoplastic neoplasm, and a careful microdissection is required to produce a high-quality map of the genetic makeup of the tumors. Introduction of gain-of-function (*Kras*) and/or loss-of-function mutations (TSGs) in these four genes in pancreatic epithelial cells in mice recapitulates many aspects of PDAC in humans and supports a possible origin of PDAC in acinar cells [106].

Autopsy studies have established that low-grade PanINs are highly prevalent in the pancreas of individuals without known pancreatic diseases and that their frequency increases with age. By contrast, high-grade PanINs are exceedingly rare except in the pancreas of subjects with PDAC [107]. A recent multicenter genetic study of high-grade PanINs from individuals without PDAC suggests that the genetic alterations that characterize these lesions are less well understood: while PanIN-3 from subjects with PDAC generally harbor alterations in the three major TSG involved in this tumor, the lesions from subjects without PDAC display a much lower frequency of alterations [108]. This has led to propose that many of the seemingly PanIN-3 lesions found in the pancreas of patients with PDAC correspond—in fact—to ductal cancerization, a phenomenon through which tumor cells invade the luminal aspect of the ducts [108].

This “uniform” scenario of genetic alterations is paralleled by alterations in a large number of additional genes that are mutated only at low frequency (“private”). Such a genomic landscape was first shown by Jones et al. [109] and then confirmed using massive parallel sequencing by the Australian Pancreatic Cancer Genomics Initiative [110], the Canadian ICGC study [111], and the TCGA [112]. In these studies, most case samples analyzed came from patients with resectable tumors. While we have a less comprehensive understanding of the genomics of metastatic disease, the available evidence supports the notion that metastatic spreading is associated with epigenetic rewiring.

### 3.2 Pancreatic cancer molecular subtypes

Genome-wide transcriptomic analyses have allowed dissecting the heterogeneity of PDAC. Collisson et al. used microarrays to acquire expression data from untreated, resected, PDAC cancer samples and identified three molecular subtypes: classical, quasi-mesenchymal (QM), and exocrine-like [113]. The classical subtype displayed high epithelial and adhesion gene expression profile, high levels of transcription factor GATA6, dependency on *KRAS*, and better outcome after resection. In contrast, the QM subtype showed high expression of mesenchymal genes, low levels of GATA6, and worse outcome. The exocrine-like subtype was enriched in transcripts coding for digestive enzymes and had an intermediate outcome [113]. However, there have been concerns about the true existence of the latter group, which could reflect contamination by normal exocrine tissue.

To overcome the challenge of bulk tumor analysis, Moffitt et al. applied nonnegative matrix factorization (NMF) to perform virtual microdissection of primary

and metastatic PDAC samples. With this approach, two tumor subtypes—classical and basal—and two stromal subtypes—normal and activated—were identified. The classical subtype was overlapped with Collisson's classical subtype, whereas the basal subtype was characterized by the expression of genes found also in breast and bladder basal tumors (e.g., epidermal keratins and laminins), worse outcome, and possibly more benefit from adjuvant chemotherapy [114]. The Australian International Cancer Genome Consortium project extended the analysis using RNA-Seq data from 96 PDAC with a high epithelial content (>40%) and discovered four stable classes of PC—squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine (ADEX). These four subtypes were also present in the extended set of 232 PDACs using array-based mRNA expression profiles encompassing the full range of tumor cellularity. The TCGA-PDAC study also provided support to the existence of similar subtypes [112], and together with other studies, it shows a high correlation between the transcriptomic and epigenetic subtypes [115]. The commonalities and differences between these classifiers have been reviewed recently [116]. Using microarrays and formalin-fixed paraffin-embedded tumors, Puleo et al. identified five distinct subtypes according to tumor- and microenvironment-derived signatures. This classifier included samples with low tumor cellularity. Samples with low stromal signals contained pure classical and pure basal-like subtypes. The other three subtypes were defined by the impact of high stromal content, including immune classical, desmoplastic, and stroma-activated subtype [117].

Chan-Seng-Yue et al. used LCM-purified PCs and whole-transcriptome sequencing of 248 patient tumors. In this series, each of the previously defined classical and basal-like subtypes was split into two subtypes (basal A and B and classical A and B), while a hybrid subtype emerged [104]. scRNA-Seq supported (1) the coexistence of cells belonging to multiple subtypes within a given sample and (2) the existence of a continuum of states ranging from “pure classical” to “pure basal” with the ability of cells to switch from one subtype to another [118–120].

Building on this concept, and using independent component analysis, Nicolle et al. have proposed that a continuous—rather than a discrete—strategy be used to classify tumors. Their signature has been validated in fine needle aspiration/biopsies [121]. This is an important contribution given that most patients with PDAC do not undergo surgery.

More recently, the combination of scRNA-Seq and spatial transcriptomics suggests even more complex scenarios with potential clinical relevance. Raghavan et al. have profiled metastatic samples and organoids to confirm the existence of transitional tumor phenotypes

influenced by signals from the tumor microenvironment [122]. In addition, Hwang et al. have proposed the existence of a neural-like progenitor subtype that is enriched in patients undergoing resection after receiving chemoradiotherapy, associated with poor prognosis [123].

In summary, tumor and stromal cell heterogeneity, intrinsic plasticity, selective pressures, and adaptive responses contribute to an increasingly complex scenario underlying PDAC diversity. The clinical usefulness of these analyses remains to be established in well-designed prospective studies, preferably in the context of clinical trials. Even though the molecular taxonomy of PDAC provides a great opportunity to accelerate the development of personalized medicine strategies, the scenario remains challenging.

### 3.3 The tumor microenvironment in pancreatic cancer

Tumor development is a highly interactive process involving not only the prospective neoplastic cells but also many normal host cellular components that promote or suppress clonal evolution required for tumor formation. Nonneoplastic host components continue to play key roles once the tumor is established, contributing to generate “the tumor microenvironment” (TME). In PDAC, the TME is particularly relevant as the stroma may comprise up to 90% of tumor mass [120,124]. The TME includes the extracellular matrix (ECM) and a wide variety of cell populations, such as cancer-associated fibroblast (CAFs), vascular endothelial cells, pericytes, neurons, and infiltrating immune cells.

*Cancer-associated fibroblasts.* Among all stromal cells, CAFs play a particularly important role owing to their capacity to secrete ECM proteins, proangiogenic, and immunomodulatory factors, all of which may contribute to the environment permissive for tumor growth and invasion [125]. The high-level production of collagen by CAFs, and the ability of collagen fibers to increase tissue stiffness and provide mechanical signals, has been proposed to contribute to the early and rapid dissemination of PDAC cells. The major source of CAFs in PDAC are pancreatic-stellate cells (PSCs), which occur in a quiescent state in the healthy pancreas and can also originate from the pancreatic mesothelium [126]. Quiescent PSCs accumulate vitamin A droplets; upon activation, they start to express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), desmin, glial fibrillary acidic protein, and acetylcholine receptors [127] and produce high levels of collagen and other ECM components. Moreover, pericytes, monocytes, adipocytes, mesothelial cells, and bone marrow-derived or adipose-derived mesenchymal stem cells can also transdifferentiate into CAFs [128–131].



CAF identification/isolation is limited by the lack of truly specific markers. They are often identified by negative selection of endothelial (CD31), epithelial (EpCAM), and hematopoietic (CD45) lineages. Most CAF markers (PDGFR $\alpha$ , PDGFR $\beta$ , PDPN, THY1, or  $\alpha$ -SMA) are neither unique nor uniformly expressed. In PDAC, Öhlund et al. first showed the existence of spatially restricted and functionally distinct CAF populations:  $\alpha$ -SMA+ myofibroblasts (myCAFs) are closely associated with tumor cells, whereas those that are IL6-expressing (iCAFs) are more distant. By RNA sequencing, they identified unique transcriptional signatures that implied that myCAFs are contractile and stroma remodeling, whereas iCAFs express cytokines and chemokines indicative of their immunomodulatory and inflammatory role [132]. A third subpopulation of antigen-presenting CAFs, which expresses MHCII and has the capacity to present antigen to CD4+ T cells *ex vivo*, was later identified in mouse and human PDAC [133]. It was recently suggested that wound-associated signals from the tumor niche induce mesothelial cells to acquire features of apCAFs, which exert their unique immune-regulating role by promoting Treg formation and expansion through antigen-dependent TCR ligation [134]. Another study used mass spectrometry to analyze mesenchymal cell populations in normal and tumor mouse pancreatic tissue. There, CD105 was identified as a marker for two functionally different CAF populations. Importantly, both CD105-positive and CD105-negative cells displayed myCAF and iCAF gene signatures. However, functionally, the CD105-positive population was tumor promoting, while CD105-negative CAFs were tumor-suppressive.

Together, these studies question whether CAF subtypes represent truly distinct lineages or are a “snapshot” of CAF plastic states. Indeed, CAFs have been shown to respond to a wide variety of signaling cues. For instance, upon plating in 2D, iCAFs and apCAFs may revert to acquire a myCAF phenotype [132,133]. Additionally, JAK/STAT and TGF- $\beta$  signaling act antagonistically to induce CAF heterogeneity. It has been proposed that tumor cells secrete TGF- $\beta$  that inhibits IL-1R1 expression and JAK/STAT signal activation in adjacent CAFs, therefore promoting a myCAF phenotype. On the contrary, lack of TGF- $\beta$  in distal tumor areas leads to high IL-1R1 expression, which stimulates NF- $\kappa$ B signaling and activates the JAK/STAT pathway, therefore supporting the iCAF phenotype [133]. TGF- $\beta$  and IL1 can also promote transition of mesothelial cells to apCAFs [134]. Short-term inhibition of the Sonic Hedgehog pathway resulted in depletion of myCAFs and enrichment of iCAFs in orthotopic PDAC xenografts, suggesting that Hedgehog signaling may also be important for the maintenance of myCAFs [135]. In addition,

hypoxia could synergize with cancer cell–derived cytokines to induce an iCAF phenotype [136]. CAF heterogeneity, plasticity, and topography are best being addressed through the use of sc-RNAseq and spatial transcriptomics in a wide variety of cancers, including PDAC [133,137–142].

CAFs can affect tumor progression through different mechanisms—collagen deposition, ECM remodeling, and secretion of growth factors, cytokines, and chemokines [143–145]. Despite early proposals that CAFs promote tumor progression, experimental evidence suggests more varied and complex roles. For instance, deletion of  $\alpha$ -SMA+ fibroblasts in a mouse model of PDAC accelerated tumor progression and induced immunosuppression [146]. By contrast, genetic deletion or pharmacological targeting of FAP-expressing fibroblasts reduced tumor growth in mouse models of PC [147]. These findings indicate that CAFs might have both tumor-promoting and tumor-restraining roles, and they highlight the need to better understand their function. These observations support that, from a therapeutic standpoint, rather than “deleting” the tumor stroma, the aim should be to “normalize” its activity [148].

*Immune cells* are essential components of the PDAC TME. They include tumor-associated macrophages (TAMs), CD4+ and CD8+ lymphocytes, regulatory T cells (Tregs), dendritic cells, and myeloid-derived immunosuppressive cells (MDSCs). In PDAC, TAMs, Tregs, and MDSCs prevail over T cells and dendritic cells, thus creating an immunosuppressive microenvironment [149]. By releasing cytokines, proteases, and growth factors, these cells can promote tumor cell growth and blunt antitumor immune responses [150–153].

One of the first infiltrating cells in PanINs are TAMs [154,155], and their number increases during progression to invasive cancer. Several studies have demonstrated an inverse correlation between TAM infiltration and patient prognosis in different tumors, including PDAC [150,156]. TAM-derived cytokines and chemokines, such as IL1 $\beta$ , IL8, and CCL18, can promote the epithelial–mesenchymal transition (EMT) in cancer cells by activating signaling by PAR1 [157] and TLR4/IL10 [158]. Coculture of PC cells with M2-polarized TAMs favored a fibroblastic morphology, upregulated vimentin and SNAIL expression, and increased proliferation, migration, and metalloproteinase (MMP2) and MMP9 proteolytic activity in tumor cells [158]. In addition, TAMs can modulate the efficacy of chemotherapy in PDAC, affecting the activity of cytidine deaminase, a key metabolizer of gemcitabine, thereby driving resistance to this drug in *in vivo* PDAC models [159].

Another cell type contributing to immune suppression and inflammation in PDAC are myeloid-derived



suppressor cells (MDSCs). MDSC content correlates with clinical cancer stage [160]. Through direct cell-to-cell contact with T lymphocytes, MDSCs upregulate PD-L1, which can lead to the suppression of T cell activation and self-tolerance [161]. Moreover, MDSCs can stimulate the expansion of Tregs, which again results in suppression of T cell function [162]. Therefore, efforts have been made to develop strategies to eliminate both TAMs and MDSCs and improve antitumor immunity.

PDACs are believed to be nonimmunogenic or cold, showing low infiltration of CD8<sup>+</sup> cytotoxic lymphocytes (CTLs), which localize at the tumor border or are trapped within fibrotic tissue but are absent from the tumor core. CTLs produce IFN- $\gamma$ , TNF, and cytotoxic molecules, such as perforin and granzymes, which are major effectors in tumor rejection. In PDAC, CTLs most often show minimal activation [155,163]. Both cancer cells and inflammatory cells (e.g., MDSCs) play critical roles in regulating T cell function and can drive T cell exhaustion [164]. Exhausted T cells express high levels of inhibitory receptors, including programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 protein (LAG-3), T cell immunoglobulin domain, and cytotoxic T lymphocyte antigen-4 (CTLA-4). Other characteristic of exhausted T cells is the loss of IL2 and granzyme B production and ex vivo killing capacity [165,166]. Reversing exhausted T cells and restoring antitumor potential have proven a valuable strategy to tackle cancer.

Altogether, an improved understanding of the normal and pathological stromal physiology, with CAFs and different immune cells, is essential to harness the therapeutic potential of a wide variety of drugs—including vitamin D.

#### 4. The biology of vitamin D in pancreatic cancer: experimental studies

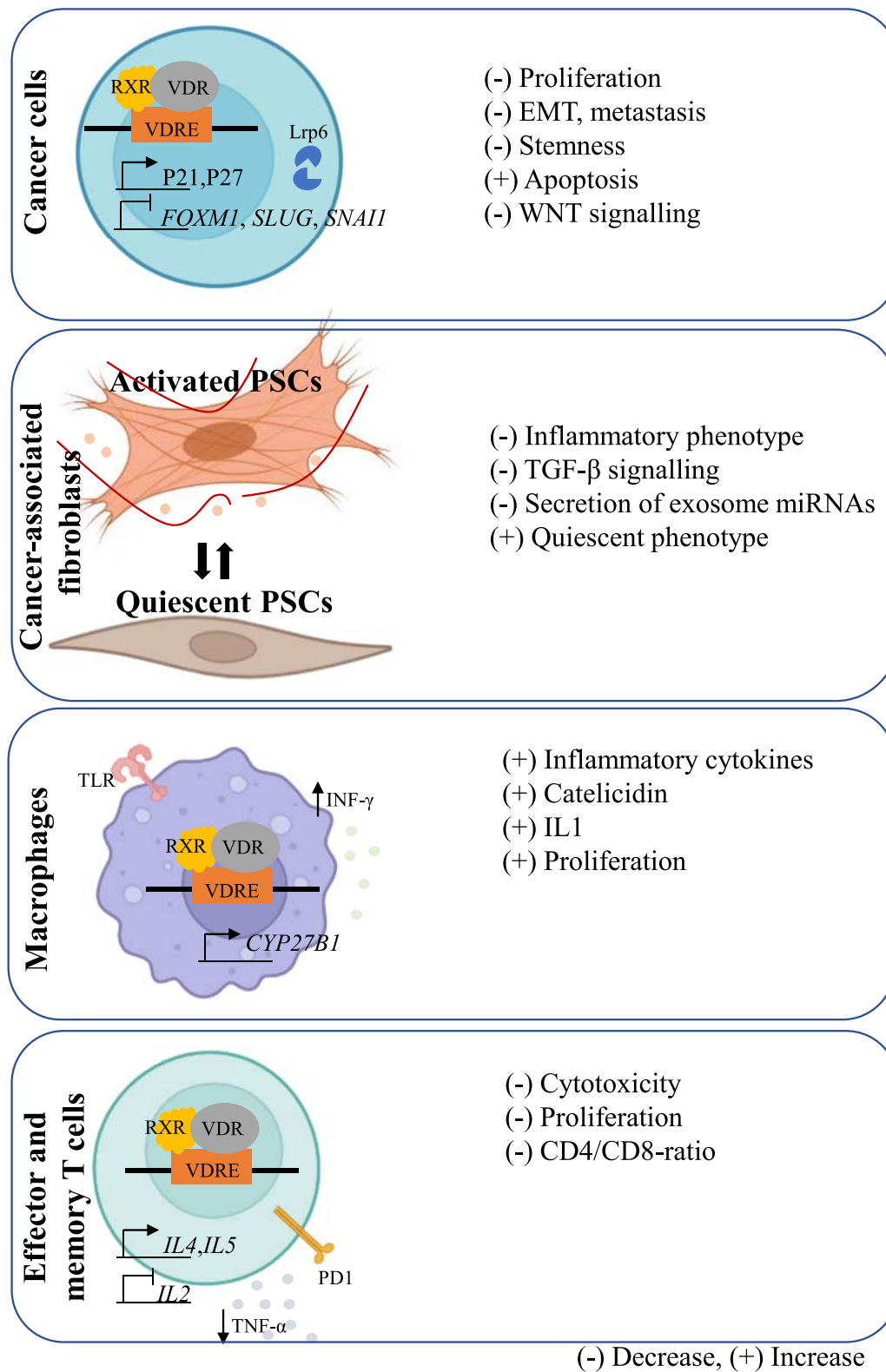
A summary of the main findings reported is shown graphically in Fig. 92.2 (Box 92.2).

##### 4.1 Vitamin D and pancreatic tumor cells

The expression of CYP27B1 and of VDR in various cell types promoted the idea that calcitriol might have autocrine and paracrine effects beyond its role in calcium homeostasis [167]. Indeed, both CYP27B1 and VDR are expressed in gut epithelial, immune, and cancer cells [168]. Recently, chromatin immunoprecipitation experiments have shown that VDR can bind to a large number of genomic sites, therefore controlling the transcription of many genes upon activation by ligand [169,170]. These experiments further support wider roles of vitamin D than initially believed.

VDR and its target gene *CYP24A1* are overexpressed in PDAC tumor cells, compared with adjacent nonneoplastic pancreas, concomitant with *CYP21A1* downregulation in tumor-associated islets. These findings suggest a dysregulation of the vitamin D system in PDAC [171]. Vitamin D can affect the biology of PC cells by different means. Early studies of the effect of vitamin D analogs on PDAC cells showed growth inhibition and G1 phase arrest [172–174], possibly through the upregulation of p21 and p27 and increased hypophosphorylated RB1 [175,176]. Moreover, the antiproliferative effects of vitamin D<sub>3</sub> have been reported to be mediated, at least in part, by inhibition of SMO and the Hedgehog (HH) pathway in a VDR-independent manner [177]. This cytostatic effect was restricted to cell types known to rely on an activated HH pathway. However, in this study, vitamin D<sub>3</sub> had no in vivo antitumor activity alone or in combination with 5-fluorouracil or gemcitabine, nor with irradiation. Beyond vitamin D, MART-10 (19-nor-2 $\alpha$ -(3-hydroxypropyl)-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>)—a calcitriol analog—was found to inhibit the EMT and block migration and invasion of BxPC3 and PANC1 cells through downregulation of SLUG and SNAIL [178]. These effects were similar to those reported earlier in colorectal cancer [179]. These antiproliferative effects in vitro are paralleled by an inverse correlation between VDR and FOXM1 expression in PDAC cells and tissues: pharmacological and genetic activation of VDR signaling lead to reduced FOXM1 expression and promoter activity which suppressed tumor stemness, growth, and metastasis [180]. An additional mechanism by which vitamin D may impair PDAC growth includes a reciprocal feedback loop between VDR and WNT signaling through the downregulation of LRP6, a canonical Wnt ligand coreceptor, and transcriptional upregulation of LDLRAP1, associated with increased LRP6 receptor internalization and degradation and reduced WNT signaling [181]. A synthetic lethality screen to identify genes whose activity is required for gemcitabine sensitivity identified the VDR, though these effects might be ligand-independent and mediated by modulating DNA damage repair. Upon VDR disruption, a reduced number of phospho- $\gamma$ H2AX and RAD51 foci in response to gemcitabine was demonstrated suggesting that VDR inhibition might cooperate with genotoxic drugs [182]. Altogether, the few studies addressing the effects of vitamin D and its analogs on PDAC cells support their ability to directly target tumor cells to impair tumor growth, stemness, and metastasis.

The studies of the in vivo administration of VDR agonists on tumor growth have yielded discordant results [180,183,184]. These discrepancies are likely due to differences in the activity of the compounds used, the responsiveness of the cellular models, and the readouts applied. In vitro and in vivo experiments have shown



**FIGURE 92.2** Overview of the biological effects of vitamin D on pancreatic cancer cells and components of the tumor microenvironment.

## BOX 92.2

## Summary of the main experimental studies

- VDR and CYP24A1 are overexpressed in PDAC cells, compared with nonneoplastic pancreas.
- In vitro studies of the effects on PDAC cells indicate that vitamin D can inhibit (1) proliferation through p21 and p27, via the SMO/Hedgehog pathway and via a reduction of Wnt signaling through increased degradation of its canonical ligand LRP6 and (2) EMT, migration, and invasion via downregulation of SLUG and SNAI1.
- In vivo studies have yielded discordant results with significant antitumor activity being observed in some cases. Discrepancies might be due to heterogeneity in the cellular models used and differences in the activity of the compounds used.
- Vitamin D treatment of activated fibroblast attenuates the expression of cancer-associated signatures and reduces proliferation, migration, and release of protumoral exosomal miRNAs.
- 1,25(OH)<sub>2</sub>D suppresses proliferation of T lymphocytes and production of cytokines, shifting from a proinflammatory to a tolerogenic state.
- In a syngeneic PDAC model, calcipotriol treatment increased immune cell infiltration and decreased T cell exhaustion and amount of Tregs.

the synergistic effect of gemcitabine and calcitriol, mediated by increased apoptosis and inhibition of Akt signaling in Capan-1 cells [185].

## 4.2 Vitamin D and the pancreatic tumor microenvironment

In the following, we describe current knowledge on the impact of vitamin D on cancer-associated fibroblasts and the immune TME compartment.

### 4.2.1 Cancer-associated fibroblasts

Most of the studies investigating the effect of vitamin D on the pancreatic TME have focused on PSCs and CAFs. Therapeutic strategies that can revert or reprogram activated PSCs to their quiescent state may hold promise for more effective treatment options alone or in combination with agents targeting the tumor cell compartment. An important study showed that calcipotriol induced transcriptional reprogramming of activated PSC toward a quiescent state, suppressed secretion of inflammatory cytokines and growth factors, increased gemcitabine treatment efficacy in vitro, and improved survival in a murine model of PDAC. RNA sequencing of preactivated, culture-activated, and cancer-associated PSCs identified signatures associated with activation and/or cancer. Calcipotriol reduced the expression of lipid-storage genes and upregulated cell proliferation and adhesion [186]. These authors also found an upregulation of VDR signaling in activated PSCs and CAFs. Treatment of preactivated and activated PSC with calcitriol showed an inhibition of activation and suppression of cancer-associated signatures and reduced expression of negative regulators of

angiogenesis—such as Thbs1—and decreased TGF- $\beta$  signaling, in agreement with prior studies showing a cross-talk with the VDR [187]. VDR activation also attenuated PSC activation, inflammation, and fibrosis upon caerulein-induced pancreatitis. Altogether, these results strongly support a role of VDR in the regulation of PSC quiescence/activation both in vitro and in vivo. To explore the ability of calcitriol to modulate the tumor-stroma cross-talk, conditioned medium from activated PSC grown in the presence of calcitriol was added to tumor cells, resulting in reduced EMT activity, proliferation, and chemoresistance. When KPC mice, harboring a mutant *Kras* allele and *Trp53* inactivation in the pancreas, were treated with calcitriol and gemcitabine, an increased intratumoral concentration of gemcitabine was demonstrated, associated with a significant decrease in tumor volume and tumor-associated fibrosis [186]. This pioneered the potential benefit of stroma remodeling rather than stroma depletion. A subsequent study confirmed that calcipotriol reduced the release of IL-6, LIF, and PGE2 but had variable effect on  $\alpha$ SMA or podoplanin expression. Consistently, calcipotriol reduced CAF proliferation and migration. However, there were no significant effects on the capacity of CAFs to modulate the contractility of the collagen matrix. In a 3D coculture system including CAFs, PDAC cells, and T lymphocytes, calcipotriol upregulated PD-L1 expression and dampened the proliferation and functionality of CD8<sup>+</sup> T cells, indicating that VDR signaling can impact broadly on the tumor ecosystem [188]. A recent study showed that VDR activation abrogated the release of protumoral exosomal miRNAs (miR-10a-5p), affecting PDAC cell proliferation and migration [189].

#### 4.2.2 The immune TME compartment

Enzymes involved in vitamin D metabolism and the VDR are expressed in a wide variety of immune cells including lymphocytes, monocytes, macrophages, and dendritic cells [190,191]. Following the recognition that vitamin D can modulate the activity of the innate immune system in tuberculosis [192], its role in monocyte and macrophage activation emerged.  $1,25(\text{OH})_2\text{D}$  produced by monocytes/macrophages can exit the cell, suppress the proliferation of T lymphocytes, and modulate the production of cytokines and T cell differentiation, resulting in a dramatic shift from a proinflammatory to a tolerogenic state. The effects of  $1,25(\text{OH})_2\text{D}$  on the differentiation, proliferation, and functions of CTL are likely mediated by both direct activation of VDR and changes in cytokines signaling via T helper cells [193,194]. Other studies have reported that vitamin D can also affect the homing of T cells to specific tissues, such as lymph nodes [195].

Few studies have investigated the effects of vitamin D on the immune component of the PDAC-TME. A recent report showed that calcipotriol improved viral delivery and replication, increased immune cell infiltration, and decreased T cell exhaustion and Tregs in a syngeneic PDAC model using oncolytic viroimmunotherapy with recombinant orthopoxvirus (CF33) [196]. Increased CD8+T cell infiltration upon vitamin D treatment has been described in breast [197] and melanoma lung metastasis models [198]. However, the contribution of stromal cells to the effects on T cells has not been fully dissected [188]. PDAC cells can induce changes in intracellular  $\text{Ca}^{++}$  signaling and NF- $\kappa\text{B}$  activation that could be reverted by calcitriol in a SMAD4-dependent manner (PMID: 32679840). However, other studies in other tumors suggest tumor-promoting effects of calcitriol through enhanced M2 macrophage polarization, leading to increased cell migration.

Overall, the paucity of studies in PDAC calls for additional research before the full potential of modulation of VDR signaling is understood.

### 5. Vitamin D and prognosis in patients with PC

Most PC patients are diagnosed with locally advanced or metastatic disease, and only 20% of patients are candidates for radical surgery, which is the only option for cure. The 5-year survival rate of patients who are candidates for surgical resection is close to 25%. An important feature of PC is that even small tumors (<2 cm) are often metastatic, suggesting that PC becomes a systemic disease even at early stages [107,199].

The survival of patients with PC has increased slightly in the past decade, but there is a need for

breakthroughs in early diagnosis and treatment to reduce mortality. The lack of sensitive and specific tumor markers that would allow detecting the disease early makes screening for PC remains extremely challenging.

The association of low vitamin D levels with prognosis in patients with PC has been investigated by several studies with different designs, reaching variable degrees of evidence (see Table 92.2 for further details). Ecological studies support an association between vitamin D and higher survival rates in cancer patients. Five-year survival rates south of  $50^\circ\text{N}$  were 20%–50% higher than those near  $55^\circ\text{N}$ , using survival data from cancer patients (among them PC) diagnosed from 1990 to 1994 from nine European countries (Eurocare-3 study) [45]. Other studies have investigated vitamin D as a prognostic factor in PC patients using different biomarkers such as vitamin D, DBP levels, VDR or CYP24A1 expression, and SNPs tagging the VDR gene.

#### 5.1 Vitamin D levels

Studies have largely used plasma  $25(\text{OH})\text{D}$  levels as a preferred measurement because it has a 2-week half-life and it reflects both dietary intake and endogenous production [200]. However, the results are inconsistent (Table 92.2).

*Direct association.* Cho et al. [78] reported that in patients with advanced PC, deficient vitamin D levels were associated with worse prognosis (HR = 1.99,  $P$ -value = .01) in a multivariate analysis considering season and BMI as confounding factors. In a prospective study of 493 patients with PC from five large US cohorts, Yuan et al. [79] found that patients with sufficient levels of prediagnostic  $25(\text{OH})\text{D}$  had a 35% lower hazard for death compared with those who were vitamin D deficient. This association was even stronger among patients with blood collected within 5 years of diagnosis, with an HR of 0.58 (95% CI, 0.35–0.98).

*No association.* Van Loon et al. [81] found that baseline  $25(\text{OH})\text{D}$  levels were not associated with either progression-free survival or overall survival in patients with advanced PC who received systemic chemotherapy. McGovern et al. [83] found that baseline  $25(\text{OH})\text{D}$  levels did not associate with PFS or OS, either. Similar findings were reported by Haas et al. [82] in a prospective single-center study of pretreatment levels of  $25\text{-OH vitamin D}_3$  in patients with advanced PC and by Von Hoff et al. [84] in a post hoc analysis of the phase III MPACT trial in patients with metastatic PC. In the ATBC cohort study, including Finnish smoker males who developed PC, results suggest an improved survival for patients with higher serum  $25(\text{OH})\text{D}$  levels, but the association was not significant [80]. Considering



the natural history of PC, it is conceivable that vitamin D may have a limited impact on outcome once patients are diagnosed.

## 5.2 Vitamin D receptor

VDR influences the expression of several hundred genes, so that vitamin D and its effectors may affect up to 3%–5% of the human protein-coding genome [201]. The role of VDR in PC has been explored at the germline level and by assessing its expression in the tumor.

*Germline genetic variants.* The association of SNPs tagging VDR with overall survival has been assessed in several studies, with contradictory results. In 493 PC patients from five US prospective cohorts who were genotyped for 36 tagging SNPs in the VDR gene  $\pm 20$  kb, Yuan et al. [79] did not find any association with overall survival (OS). In contrast, Innocenti et al. [85] reported that patients with advanced PDAC treated with chemotherapy who were homozygous for the GG allele of the VDR-rs2853564 SNP had a significantly longer survival (10.6 months) than those who were heterozygous (8.2 months) or homozygous for the AA allele (6.6 months). An interaction with high pretreatment levels of 25(OH)D as well as with gemcitabine treatment was reported. The authors suggested that rs2853564 might be clinically relevant primarily in the presence of adequate vitamin D supply; this variant appears to associate with reduced IRF4 binding and increased transcriptional activity in reporter assays. Li et al. [77] found that VDR-rs2228570 significantly associated with pathological differentiation and VDR-rs1544410 with TNM classification. These findings need to be replicated in independent studies.

*VDR expression in PC:* Using IHC, Wang et al. [86] found that VDR was detected in normal pancreas, with higher expression in acinar and ductal cells than in the stroma. VDR expression was higher in well-differentiated and in smaller ( $<25$  mm) tumors (64% vs. 26%). Low VDR expression in tumors was associated with a poor prognosis. They hypothesize that VDR expression in the stroma could largely depend on the activation status of pancreatic stellate cells, acting as a regulator of the cross-talk between tumor and stroma [86]. Consistently, a 3.7-fold higher expression of VDR mRNA was found in PDAC compared with adjacent pancreas tissue [171].

## 5.3 Vitamin D–binding protein levels

DBP is involved in vitamin transport in plasma, among other functions. Abulaizi et al. [202] found that DBP plasma levels were significantly diminished in PC patients. Iuga et al. [87] analyzed nine patients and

found higher DBP expression in tumor than in normal tissue. Furthermore, they found an association of low DBP expression with reduced survival.

## 5.4 CYP24A1 expression

CYP24A1 is an essential component of vitamin D metabolism, through inactivation of 1,25(OH)<sub>2</sub>D by hydroxylation; it has been reported to be overexpressed in PC and in nonneoplastic adjacent areas from surgical resection specimens, with no association with survival. Trends for association with differentiation and lymph node metastases were reported [88].

## 6. Vitamin D intervention studies in patients with PC

Two clinical trials found significantly reduced all-cancer incidence for individuals taking vitamin D in addition to calcium, compared with placebo or calcium alone [203,204]. Clinical trials have also found that vitamin D administration is associated with reduced levels of biomarkers of inflammation [205]. However, a recent *post hoc* analysis of data from the Vitamin D Assessment trial concluded that a high-dose vitamin D supplementation prescribed monthly for up to 4 years without calcium may not reduce cancer risk [206]. None of these intervention studies has investigated specifically the incidence of PC, likely due to its low incidence.

The effects of the administration of vitamin D analogs in combination with chemotherapy have been assessed in several clinical trials (see Table 92.3 for further details).

*Seocalcitol*, also known as EB1089, was administered to patients with inoperable PC in a phase II clinical trial [89] based on promising preclinical evidence [86]. While well tolerated (the most frequent toxicity being dose-dependent hypercalcemia), there was no antitumor effect nor an improvement of patient survival.

*Calcitriol* (1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>). A phase II clinical trial including 25 patients with unresectable locally advanced or metastatic PC who received docetaxel concurrently with high-dose oral calcitriol showed a modest increase in time to progression (from 1.5 to 3.8 months) without improvement of OS [90].

*Vitamin D<sub>3</sub>.* High-dose vitamin D<sub>3</sub> (180,000 I.U. on day 1, followed by 4000 I.U. per day for 60 day) was administered to PC patients with vitamin D deficiency in a randomized phase 3 clinical trial (NCT03472833). The trial was terminated due to poor recruitment and follow-up during the Covid-19 pandemic, and the results of the study have not been reported.

*Paricalcitol (19-Nor-1 $\alpha$ ,25-dihydroxyvitamin D<sub>2</sub>)* is a synthetic noncalcemic, non-phosphatemic vitamin D analog. Nine clinical trials registered in Clinical Trials.gov (accessed August 22, 2022) evaluated the effect of paricalcitol in combination with chemotherapy in PC patients using different designs. NCT03331562, a double-blind randomized placebo-controlled phase II clinical trial, found no difference in survival in patients who had advanced PC and received systemic chemotherapy [81]. NCT03300921 was terminated because of results suggesting that paricalcitol may be harmful to a subset of PC patients and feasibility issues to subtype patients in the short time frame before surgery. NCT03519308 did not meet the accrual goal. Three clinical trials (NCT04617067, NCT03883919, and NCT03415854) are active although not recruiting, and NCT03520790 and NCT04524702 are currently recruiting patients with stage IV PC and advanced/metastatic PC, respectively.

*Dietary supplementation (curcumin, vitamin D, vitamin K2, vitamin K1, B-6, high selenium broccoli sprouts, epigallocatechin gallate, L-carnitine, garlic extract, genistein, zinc amino chelate, mixed tocopherols, ascorbic acid, D-limonene).* A feasibility pilot phase I trial (NCT02336087) is evaluating gemcitabine/abraxane plus metformin and a dietary supplementation including vitamin D in patients with unresectable PC. The study is active, although not recruiting, and no results have been posted.

## 7. Conclusions

The broad biological effects of vitamin D, and the evidence of associations with better health status, render vitamin D an attractive subject of study in PC. The studies reviewed here provide conflicting evidence on the association with risk, prognosis, and its possible effects in the postdiagnosis setting. Among the reasons of this lack of consensus among studies are the different geographical settings and UV light exposures, population heterogeneity, the distinct vitamin D-related compounds measured, the drugs/doses used for supplementation, as well as the heterogeneity of study designs, data collected, and analytical approaches applied.

Current knowledge supports the notion that reducing the proportion of individuals with inadequate levels of vitamin D in the general population may impact on reducing the risk of PC, possibly by lowering the impact of risk factors associated with this tumor. However, formal evidence needs to be acquired, and intervention studies should be the best approach to address these questions. The latter should focus on the PC high-risk subjects with insufficient/deficient levels of vitamin D, also possibly considering their genetic profile. On the

other hand, the advanced stage at which patients with PC are diagnosed, their advanced aged, and the low performance status of many patients complicate the study of the impact of vitamin D supplementation in the post-diagnosis setting. The experimental work conducted in the past few years strongly supports the notion that vitamin D may effectively modulate the activity of the tumor stroma, and this could contribute to enhance the antitumor activity of the immune system, increase drug delivery, and improve patient outcome. This requires the conduct of well-designed clinical trials incorporating a panoply of biomarker analyses that allow acquiring mechanistic insight to optimize the use of this hormone.

## 8. Summary points

- Overall, there is not sufficient evidence supporting that primary, secondary (e.g., screening for vitamin D deficiency and vitamin D supplementation), or tertiary prevention strategies should be applied to reduce PC incidence.
- In vitro and in vivo studies support the notion that vitamin D can affect the phenotype and biological behavior of both tumor cells and the tumor microenvironment.
- More work is needed to establish the potential of vitamin D to reduce the medical impact of PC.

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# Sunlight, skin cancer and vitamin D

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## OBJECTIVES

- Present the ways in which skin can adapt to UV exposure and note the similarity of the action spectra for vitamin D synthesis and skin cancer production.
- Describe the main forms of UV-induced DNA damage, how the DNA damage is repaired, and the role of UV-induced immune suppression in photo-carcinogenesis.
- Outline the ways in which the vitamin D system in skin, comprising vitamin D-related compounds and receptors, modify skin cancer development and outcomes.
- Present the proposed mechanisms by which vitamin D compounds reduce UV-induced DNA damage with emphasis on reductions in reactive oxygen and nitrogen species, increased energy provision, increased DNA repair proteins and activity and the receptors involved.
- Discuss the role of vitamin D compounds in modifying UV-induced inflammation and immune suppression and other factors that contribute to skin cancer development.

pressure and of course, vitamin D synthesis [1]. The downside is that sunlight exposure is a major factor in the development of skin cancers, both keratinocyte and melanocyte-derived cancers [2]. Solar ultraviolet (UV) radiation contains UVA, UVB, and UVC. UVC ( $\lambda$  100–290 nm) is mostly removed by the ozone layer. UVB ( $\lambda$  290–320 nm), normally around 5% of total UV in sunlight and UVA ( $\lambda$  320–400 nm), comprising the remaining 95% of solar UV radiation, are major skin carcinogens that penetrate the epidermis and part of the dermis [3]. UV radiation produces DNA damage directly and via reactive oxygen and nitrogen species and causes immune suppression. While mild immune suppression may actually have benefits in reducing the risk of some autoimmune diseases [4], the combination of DNA damage and immune suppression is essential to the formation of skin neoplasms [5,6].

## 1.1 Sunlight and skin cancers

The most common type of skin cancer, basal cell carcinoma (BCC), is derived from keratinocytes, as is the rather less common, but potentially metastatic, squamous cell carcinoma (SCC), while the uncommon, but potentially deadly cutaneous malignant melanoma (CMM) is derived from melanocytes [7]. The incidence of skin cancers increases with age. For countries with large Caucasian populations living in tropical and temperate areas, such as the United States and Australasia, skin cancers are not only the most numerous type of cancer, but also the most costly to the health system [7,8].

The relationship between sun exposure and development of skin cancers is complex. Greater total

## 1. Introduction

Sunlight is essential for life on earth. Apart from providing energy for photosynthesis, light and heat, in human physiology, sunlight exposure has many positive effects on circadian rhythm and mood, blood

occupational or leisure UV exposure increases the risk of keratinocyte-derived skin cancer [7]. In general terms, increased sun exposure leads to increased SCC, whereas the relationship is less direct with BCC [7,9]. There is some evidence in European populations that cumulative sun exposure hours increase the risk of BCC up to a plateau, with little further increase in risk with further exposures, although the relationship depends on skin type [9]. Sun exposure that is “intermittent,” which includes high exposures at weekends or holidays, tends to be associated with increased risk of CMM and to some extent BCC [7]. The risk of all skin cancers is increased by sunburn at any age, with the largest effect on melanoma incidence, followed by BCC and then SCC [7,10]. There is epidemiological data showing that the risks of all major skin cancers are reduced by half in relatively light-skinned people who migrate from a low UV environment to a high solar UV environment, like Australia, after the age of 10 years, compared with people who lived all their lives in a high solar UV environment [11]. Having light skin, Fitzpatrick’s type I or II [12] increases the risk of all types of skin cancer, while ability to tan reduces the risk, mostly for SCC followed by BCC and then melanoma [7]. SCC and BCC tend to occur on chronically sun-exposed areas such as the face, ears, neck, and back of the hands, while melanomas occur in these areas but also on shoulders, backs, and limbs and sometimes on palms and soles [13], though the pathogenesis of melanoma in non-sun-exposed areas appears to be different [14,15].

## 1.2 Adaptation to UV

As sun exposure gradually increases after winter, there are several mechanisms that allow skin to adapt to UV exposure and protect against UV-induced damage. These include an increase in production of melanin, which is transferred to keratinocytes and provides a shield over the nucleus [16]. Even in people who do not tan well, the outer cornified layer of the epithelium (stratum corneum) can thicken markedly as a result of UV exposure, leading to a reduction in UV transmission [17]. Over recent years, accumulating evidence, also discussed in Chapter 25 (Bikle & Demay), indicates that there is another UV-adaptive mechanism in skin: the vitamin D system, including the vitamin D receptor (VDR) and local vitamin D synthesis and metabolism that generates 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}_3$ ) and other related compounds [18–21]. These vitamin D compounds, working at least in part via the VDR, contribute to reductions in UV-induced DNA damage and to reduced UV-induced immune suppression, at least in some mouse models, leading to reduced photo-carcinogenesis in mice [2,22–26].

## 1.3 Similarity of action spectra for vitamin D synthesis and skin cancer

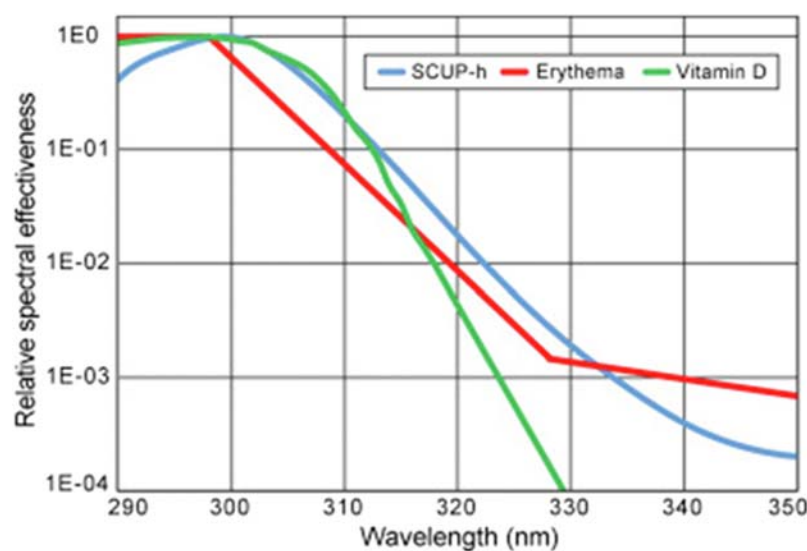
It has been noted by Thompson and colleagues [27] that while the action spectrum for pre-vitamin D synthesis may differ slightly at low wavelengths from that for erythema, the empirically derived Skin Cancer Utrecht-Philadelphia-human (SCUP-h) spectrum [28] is remarkably similar to that for vitamin D synthesis *in vitro*, at least in the UVB range up to 315 nm (Fig. 93.1) [27]. Recent work on the UV action spectrum to increase blood 25-hydroxyvitamin D<sub>3</sub> ( $25(\text{OH})\text{D}_3$ ) in human subjects indicates a left shift of around 5 nm [29]. If anything, this increases the overlap of the vitamin D and skin cancer action spectra. Any exposure to UVB, necessary to convert 7-dehydrocholesterol into previtamin D (see Chapter 3), results in DNA damage, measurable as excised thymine dimers (see Section 3) in urine [30]. Nevertheless, in the middle of the day, when the UVB to UVA ratio is around its highest level, and a reasonable body surface area is exposed, equivalent to wearing short sleeves and shorts, UV exposure times for vitamin D synthesis are much less than for erythema for most light skin types [31]. Due to the degradation of pre-vitamin D and vitamin D by further exposure to UV, short frequent exposures to UV are more efficient for vitamin D synthesis and at the same time reduce erythema risk [31].

As also discussed in Chapter 25 and in this chapter, there is evidence that the vitamin D system, vitamin D compounds, and possibly the unliganded VDR [18,32] contribute to protection from all the major types of skin cancers (SCC, BCC, and CMM) [2,33].

## 2. UV-induced DNA damage, repair, and immune suppression

### 2.1 Direct DNA damage by absorption of UV

Two primary DNA lesions formed in human skin as a direct consequence of absorbing energy from UV photons are cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts. CPDs occur when UVB cleaves DNA between carbon-5 and carbon-6 of adjacent pyrimidines. Subsequent dimerization of these pyrimidines produces a stable ring structure (Fig. 93.2). The majority of CPDs occur at thymine–thymine pairs resulting in thymine dimers. Thymine–cytosine and cytosine–cytosine dimers are also formed, at a rate proportional to that of thymine–thymine dimers, but with lower frequency, as they require more energy to produce [34,35]. The stable ring configuration of CPD and the relatively small degree of distortion of the DNA strand make this structure hard to detect and repair [36,37]. 6-4 photoproducts have a stable bond between positions 4 and 6 of adjacent pyrimidines and



**FIGURE 93.1** Action spectra describing UV dose-dependent response relationships for skin cancer, erythema, and cutaneous vitamin D production. SCUP-h, Skin Cancer Utrecht-Philadelphia-human.

occur at a much lower frequency than CPD [34]. These 6-4 photoproducts cause more helix distortion than CPD, which may be one reason that they are repaired more rapidly after UV exposure [36,37]. For these reasons, CPDs are the most frequent promutagenic DNA photoproducts in human skin [38]. Although the high energy of UVB and its ready absorption by DNA is considered necessary for the production of CPD, it has been reported that CPD can be produced by less energetic UVA radiation, apparently by direct absorption [35,39,40]. Generation of CPD may actually explain a considerable part of the mutagenic effects of UVA [41].

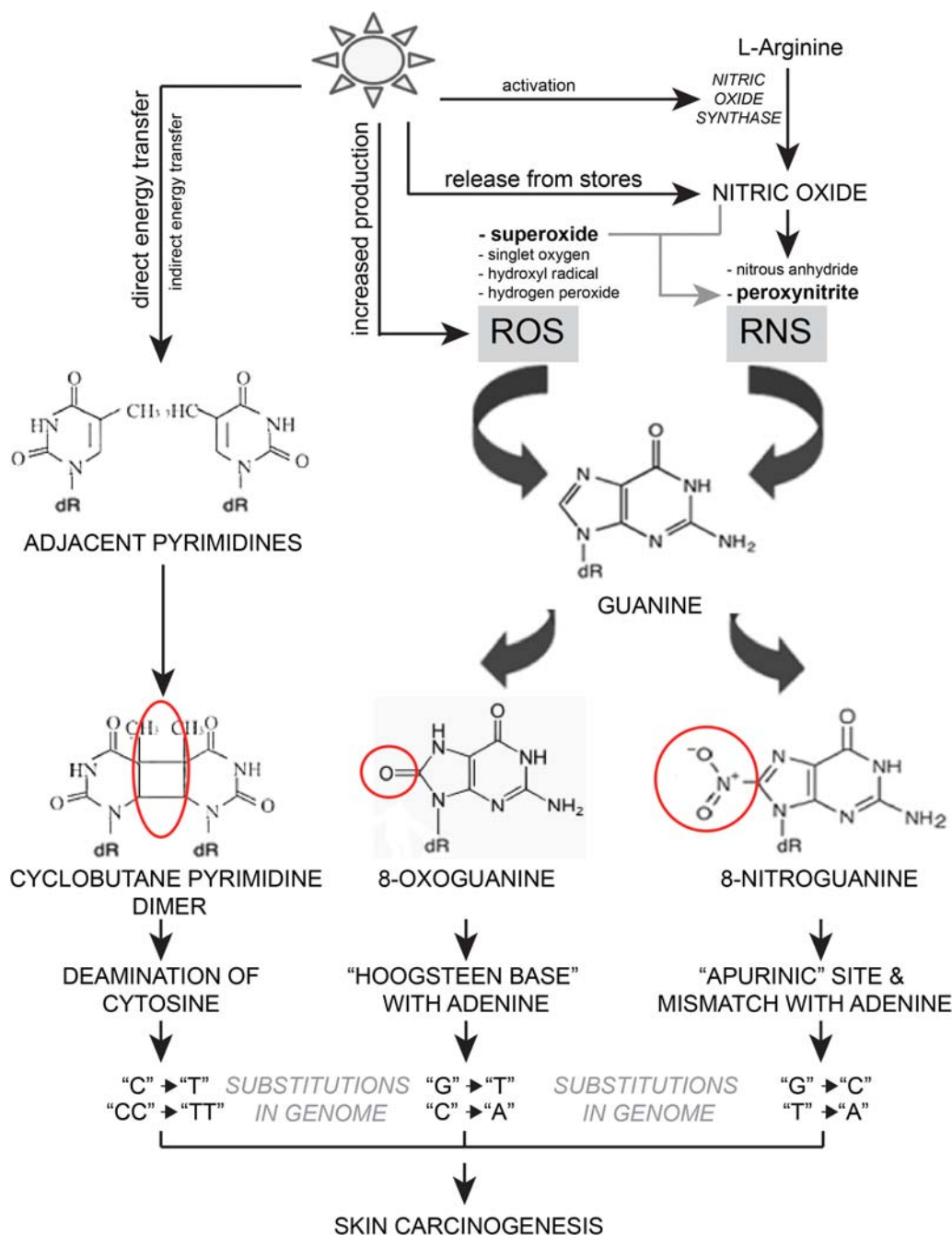
Inefficient repair of CPD and 6-4 photoproducts can result in mutations in the DNA sequence known as UV “signature” mutations [38,42]. If there are unrepaired UV-induced lesions in DNA during the S-phase of the cell cycle, the error-prone DNA polymerase-eta (a translesion polymerase) mainly inserts an adenine (A) by default, opposite lesions on the template strand that it cannot interpret, such as bulky dimers. This means that photoproducts, mainly CPD and less commonly 6-4 photoproducts, generate a characteristic C to T transition mutation. If the lesions are CC CPD, a CC to TT transition occurs, because two A residues are placed opposite the dimer by default in the place of two guanine (G) residues [43]. Although considered to have less mutagenic potential than other dimer types, there is evidence that the most common CPDs, thymine dimers, are indeed mutagenic [35,41]. CPDs are responsible for most of the mutagenic effects of UVB [37,38].

## 2.2 Indirect DNA damage by UV

UV exposure (both UVB and UVA) also generates reactive oxygen species, such as superoxide,

singlet oxygen, and the hydroxyl radical [44]. Nitric oxide and its products are also greatly increased in skin as a result of UV exposure, mainly through enzyme-independent release from preformed stores, for example, by UVA photodecomposition of nitrosothiols and nitrite, and partly through increases in inducible nitric oxide synthase (iNOS) [45,46]. Two major intermediates of the nitric oxide pathway, nitrous anhydride and peroxynitrite, are powerful nitrating and oxidizing agents formed when nitric oxide combines with oxygen or superoxide, respectively [47]. Oxidation of the primary amine of guanine generates predominantly 8-oxo-7,8-dihydro-2'-deoxyguanine (8-oxoguanine) (Fig. 93.2), which is typically associated with G to T transversions, and thus mutagenic if not properly repaired [44,48]. Reactive nitrogen products, including peroxynitrite, can also combine with guanosine to form 8-nitroguanine [49] (Fig. 93.2). Residues of 8-nitroguanine undergo rapid depurination to form a basic sites, which are promutagenic [50]. Deamination of purine and pyrimidine bases by nitration of primary amines by nitrous anhydride leads to DNA strand breaks [51]. DNA replication across noncoding sites of unrepaired base modifications can result in deletions, base mispairing, or substitutions where adenine is the default base added [43]. Nitrative deaminations generate C to T transition mutations in isolated DNA [51]. G to T transversions are typically associated with the 8-oxoguanine lesions in the tumor suppressor protein p53 and correlate with malignant transformation in skin photo-carcinogenesis [38]. Predominantly, C:G to T:A transversions, which cause inactivating mutations in p53, have also been reported in isolated DNA exposed to peroxynitrite [52].





**FIGURE 93.2 DNA damage by UV exposure.** Cyclobutane pyrimidine dimers are formed by direct absorption of UVB or UVA photons or less commonly by energy transfer from excited electrons. UV generates reactive oxygen and reactive nitrogen species, which oxidize or nitrate guanine bases. If improperly repaired, all of these changes in DNA can lead to mutations. DNA (6-4) photoproducts and single- or double-strand breaks are not shown. Reproduced from Ref. [27] with permission.

Indirect mechanisms may also contribute to generation of CPD. CPDs, termed "dark CPDs," are generated in melanocytes for 3 h after exposure to UVA [53]. These "dark CPDs" could be generated when UVA-induced reactive oxygen and nitrogen species excite an electron in melanin to create a quantum triplet state that has

the energy of a UV photon and is thus able to induce CPD by energy transfer [53]. The authors observed this effect in melanocytes, but proposed that a similar process and time course might be observed in keratinocytes [54,55], as keratinocytes too could contain melanin as a result of transfer of melanosomes from melanocytes [16].

## 2.3 DNA repair

DNA repair pathways include nucleotide excision repair (NER), base excision repair (BER), mismatch repair, double-stranded break repair, and photoreactivation. The pathway that is activated depends on the primary DNA lesion and the species [43]. Photoreactivation, which repairs CPD on a single strand of DNA, relies on an efficient enzyme, photolyase, which uses energy from the violet/blue end of visible spectrum. Photolyase does not seem to be active in placental mammals [56].

### 2.3.1 Nucleotide excision repair

In placental mammals, including humans, UV-induced pyrimidine dimers and 6-4 photoproducts are predominantly repaired by nucleotide excision repair (NER), a complex mechanism involving around 30 proteins [57]. The significance of this repair pathway in skin cancer is highlighted by the inherited recessive disorders xeroderma pigmentosum and Cockayne syndrome, in which there are mutations in genes encoding DNA repair or repair-related proteins, leading to defects in an aspect of nucleotide excision repair [58]. These patients have up to a 1000-fold increased incidence of skin cancer [59]. The first step in NER is carried out by damage recognition proteins which have the ability to identify small areas of damaged DNA from the vast amount of undamaged DNA. Two sub-pathways of NER have been identified, transcription-coupled repair and global genomic repair. The main difference between transcription coupled repair and global genomic repair lies in the initial damage recognition steps. Transcription-coupled repair involves removing lesions from the active transcribing strand of DNA, with RNA polymerase II as the initial damage sensor. The global genomic repair pathway repairs photolesions from any position of the genome through use of several damage recognition proteins as the initial damage sensor [57,60]. Repair of CPD is surprisingly slow, with appreciable numbers of CPD apparent > 24 h after UV exposure [35,37] and with even slower repair being evident for UVA-induced CPD compared with those produced by UVB [37]. This may be because as well as being pro-mutagenic, ROS produced by UVA inhibit nucleotide excision repair [61]. Overproduction of nitric oxide can inactivate DNA repair enzymes [61]. Other reactive oxygen/nitrogen molecules, such as peroxynitrite, are also pro-mutagenic and preferentially inhibit the excision and ligation steps of nucleotide excision repair [62], while conversely, NOS inhibitors enhance DNA repair mechanisms [63].

The first process in DNA repair is recognition of the lesion. Xeroderma pigmentosum (XP) complementation group C protein (XPC) is a versatile sensor of DNA structural distortions that, in turn, are critical to trigger repair [57]. The XPC protein complex is not particularly effective,

on its own, for detecting CPD, as opposed to other types of damage, probably because of the relatively small distortion of the DNA helix by CPD. The initial detection of CPD depends on UV-damaged DNA-binding protein (UV-DDB), which is a heterodimer of DDB1 (XPE-binding factor) and DDB2 (part of XPE) and associated proteins. In particular, DDB2 has been shown to directly interact with UV-induced dipyrimidine photoproducts. This binding of UV-DDB, in turn, recruits XPC and the rest of the nucleotide excision repair pathway [64]. Human cells with XPE mutations show profound defects in the repair of CPD [57]. Transcription-coupled repair is initiated by a stalled RNA polymerase II, which, in turn, recruits Cockayne syndrome proteins, CSA and CSB, to facilitate further assembly of the transcription-coupled NER complex [57,59].

After initial damage recognition, both pathways converge on a common step that depends on XPA as part of a much larger protein complex. The transcription initiation factor IIH (TFIIH) complex, which unwinds the damaged DNA double helix, consists of 10 protein subunits. These include two TFIIH basal transcription factor complex helicase subunits, XPB and XPD. These two proteins are involved in extending the opening of the DNA strands around the damaged lesion, but XPD is especially critical for verification of DNA damage. It is worth noting that transactivation of certain genes via the VDR is also mediated by TFIIH [65]. Full opening of the helix is further assisted by XPA, XPG, and the single-strand binding protein replication protein A (RPA). Further damage verification is carried out by XPA, which can detect nucleotides with distorted chemical structures in single-stranded DNA. The damage site in the single strand is incised at the double-strand junction by endonucleases ERCC1-XPF complex and XPG. DNA polymerase synthesizes a single DNA strand to fill the gap in the incised strand. Finally, DNA ligase seals the nick in the DNA strands [57]. This conservative and highly specific pathway ensures that the UV-damaged photolesions are removed from the genome, but if the damage is extensive, cells undergo apoptosis.

### 2.3.2 Base excision repair

Oxidative DNA damage and nitrative DNA damage are eliminated by base excision repair (BER) [44,48,66]. The repair enzyme human 8-oxoguanine-DNA glycosylase 1 (Ogg1) is less abundant in the basal layers than the superficial layers of the skin, however, which indicates that repair of oxidative damage in the dividing cells of the epidermis is less efficient [44]. Base excision repair is more rapid than NER [67]. Initial damage recognition and the removal of the damaged base are carried out by Ogg1 or alkyladenine DNA glycosylase for RNS-modified DNA [68]. After damage recognition, the same enzyme catalyzes cleavage of the N-glycosidic

bond between the base and the 2′deoxyribose sugar molecule to remove the damaged base. The removal of the damaged base from the strand forms an abasic site known as an apurinic/apyrimidinic (AP) site. The DNA backbone of the abasic site is incised by a DNA AP endonuclease or DNA AP lyase to form a 5′ single-strand nick at the AP site. The gap in the DNA strand is filled with the correct complementary base by DNA polymerase. After this, a DNA ligase completes the repair by sealing the nick in the DNA helix [48,66].

## 2.4 UV-induced immune suppression

Immune responses in the skin that would be expected to detect and prevent the development of tumors in skin are suppressed by even low or single doses of UV [63,69,70]. The importance of suppression of adaptive immunity in the pathogenesis of skin tumors was demonstrated by the pioneering work of the Kripke group. UV-induced tumors transplanted into UV-irradiated mice continued to grow, whereas they were rejected in un-irradiated mice [5,6]. The vastly increased risk of skin cancer development, particularly SCC and to some extent BCC, in immunosuppressed people, such as those who have received organ transplants [71,72], is further key evidence for a role of immune suppression in skin cancer development.

Pyrimidine dimers are important mediators of photo-immune suppression [63,73]. Exposure of UV-irradiated opossums to visible light, which activates the pyrimidine dimer repair enzyme photolyase, resulted in a reduction in CPD and in immune suppression [74]. Photoimmune suppression was also reduced in irradiated mice and in human subjects after reduction of CPD by application of encapsulated T4 endonuclease, the specific repair enzyme for pyrimidine dimers [63,73].

Exposure to UV results in a substantial reduction in numbers of Langerhans cells, which normally are involved in immune surveillance in the skin, as well as reducing the antigen-presenting ability of those remaining [70,75]. Mediators of photoimmune suppression include increased immunosuppressive cytokines such as interleukin (IL)-10 from keratinocytes and mast cells [76,77], increased proinflammatory cytokines such as IL-6 [78,79], and suppressed immune-stimulating cytokines such as IL-12 [80]. *Cis*-urocanic acid formed by UV isomerization of the photoreceptor *trans*-urocanic acid located in the outermost layers of skin also inhibits the antigen-presenting propensity of Langerhans cells [81]. The peroxidation of lipids by ROS such as peroxynitrite has been implicated in release of cytokines, which in turn modulate regulatory T cells and indeed regulatory B cells, suppressing immune responses at distant sites [77,80]. T regulatory cells help

maintain a balance between immunosuppression and autoimmunity [77]. These cells are present in skin as well as skin draining lymph nodes and therefore could also be subjected to DNA damage and oxidative stress. Antioxidant treatment has been shown to abolish immune suppression mediated by the lipid peroxidation pathway in irradiated mice [82]. Mast cells are clearly important for the development of UV-induced immune suppression since UV exposure does not suppress immunity in mast cell-deficient mice [83]. This may be partly due to migration of mast cells in skin, activated by UV-induced platelet activating factor, to B cells in draining lymph nodes. If this migration is blocked, there is no UV suppression of T cell-mediated immunity [83].

Both UVB and UVA components of sunlight are immunosuppressive in mice and humans [84–88]. Although certain wavelengths of UVA have been shown, at least in some systems, to have a protective effect against UVB-induced immunosuppression in mice [86], there is conflicting data in animals and human subjects [85–87,89]. A further complication is that UVA and UVB have interactive effects that are not just additive. When given separately to humans, low-dose UVA and UVB were not immune suppressive 3 days after exposure, but when the same overall dose was delivered as solar-simulated UV, it caused potent immune suppression [84]. Most people are normally exposed to sunlight that contains both UVA and UVB.

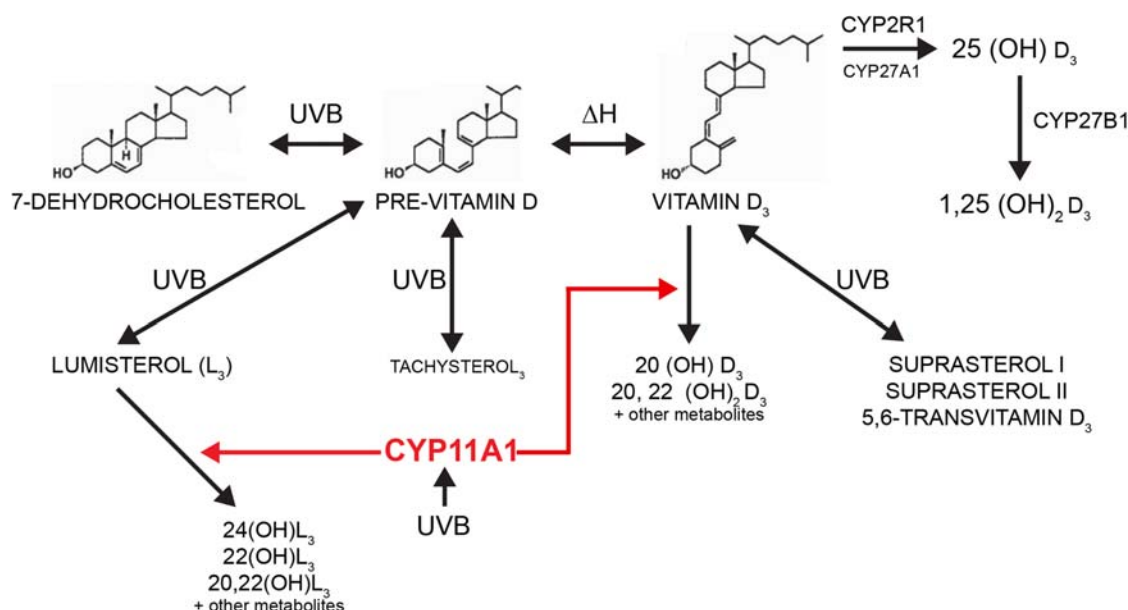
UV exposure suppresses both contact hypersensitivity reactions and delayed-type hypersensitivity reactions to viral, bacterial, and fungal antigens and causes both local and systemic suppression of the adaptive immune system [1]. This immune-suppressive effect is greater in male mice and male humans [90,91]. While excessive UV exposure promotes the development of skin cancers, mild UV-induced immune suppression may help maintain immunological self-tolerance, through the actions of T regulatory cells, UV-B regulatory cells, mast cells, and IL-10 [1]. In contrast to its effect on adaptive immunity, UV exposure boosts innate immunity, possibly in part, due to production of vitamin D and its metabolites [92,93].

In summary, as outlined before, both UV-induced DNA damage, of various types, if not properly repaired, combined with UV-induced immune suppression together lead to photo-carcinogenesis [43].

## 3. The vitamin D system and skin cancers

### 3.1 Synthesis and metabolism of vitamin D compounds in skin

The photosynthesis of vitamin D<sub>3</sub>, via the intermediate, previtamin D<sub>3</sub>, which, at body temperature,



**FIGURE 93.3** Production and metabolism of vitamin D<sub>3</sub> and related compounds in skin. Previtamin D is synthesized when the B-ring of 7-dehydrocholesterol is broken upon the absorption of a photon of UVB. At body temperature, previtamin D is converted to vitamin D<sub>3</sub>. Continued absorption of UV photons by previtamin D or vitamin D<sub>3</sub> results in conversion to overirradiation products such as lumisterol<sub>3</sub>, tachysterol<sub>3</sub>, or suprasterols or 5,6-transvitamin D<sub>3</sub>. Vitamin D<sub>3</sub> is converted in skin to 25-hydroxyvitamin D<sub>3</sub> by CYP2R1 (or possibly CYP27A1) and then to 1,25-dihydroxyvitamin D<sub>3</sub> by CYP27B1. The cholesterol side-chain cleavage enzyme CYP11A1 is also expressed in skin and upregulated by UV. It can convert vitamin D<sub>3</sub> into 20(OH)D<sub>3</sub> and at least 10 other products. CYP11A1 can also convert lumisterol<sub>3</sub> into 24-hydroxylumisterol<sub>3</sub> and several other lumisterol derivatives.

isomerizes into vitamin D<sub>3</sub> has been described in Chapter 3. Vitamin D<sub>3</sub> from skin is absorbed into the blood by a process that requires vitamin D-binding protein (DBP) [94]. The classical pathway for activation of vitamin D<sub>3</sub>, via 25-hydroxylase activity to 25(OH)D<sub>3</sub> and then to the active hormone, 1,25(OH)<sub>2</sub>D<sub>3</sub>, has been reviewed in Chapter 4. All these reactions have also been shown to occur in skin [95–97], including the production of 1,25(OH)<sub>2</sub>D<sub>3</sub> in human skin in vivo [96]. The production of vitamin D<sub>3</sub> and its subsequent metabolism takes many hours in skin [95]. Absorption of additional photons by pre-vitamin D leads to the generation of so-called “over-irradiation products” such as lumisterol and tachysterol [98]. Less well known is that another CYP450 enzyme, the cholesterol side-chain cleavage enzyme CYP11A1, is also active in skin, and its expression is increased by UV [99–101]. CYP11A1 also hydroxylates the side chain of vitamin D<sub>3</sub> in vivo, to produce over 10 novel products [100]. 20-Hydroxyvitamin D<sub>3</sub> (20(OH)D<sub>3</sub>) is the major product of vitamin D<sub>3</sub> metabolism by CYP11A1 [99]. 20(OH)D<sub>3</sub> is present in serum at concentrations about 20-fold less than those of the major circulating D metabolite, 25(OH)D<sub>3</sub>, compared with 1000-fold less for 1,25(OH)<sub>2</sub>D<sub>3</sub>. CYP11A1 also metabolizes lumisterol<sub>3</sub> to 24-hydroxylumisterol<sub>3</sub> (24(OH)L<sub>3</sub>) and several other related compounds, which have biological activity, at least in some systems [102] (see Fig. 93.3). This is discussed in greater detail in Chapter 6.

### 3.2 Relationships between the vitamin D system and the three main types of skin cancers

There is accumulating evidence that the vitamin D system (metabolites and receptor) in skin contributes to reductions in both keratinocyte- (BCC and SCC) and melanocyte-derived (melanoma) tumors (see also Chapter 25). The critical UV-induced alterations that result in photo-carcinogenesis—DNA damage that is not repaired properly and immune suppression—contribute to the development of all types of skin tumors. In relation to melanocytes, it appears that inadequately repaired DNA damage produced by both UVB and UVA, together with UV-induced immune suppression, contributes to the pathogenesis of melanoma, particularly on sun-exposed skin [103,104]. Inadequately repaired DNA damage in melanocytes may lead to mutations or amplifications of genes involved in a variety of growth and survival pathways, such as BRAF, Kit, and cyclin D1 [105]. Melanocytes are different from keratinocytes in that they do not proliferate much and have reduced DNA repair capacity, but tend to be more resistant to apoptosis, despite significant DNA damage. These differences may help to explain the somewhat different patterns of UV exposure associated with melanoma compared with SCC [106,107]. Studies in transgenic mice indicate an interaction between β-catenin and the vitamin D system in skin, which, when



disrupted, promotes BCC development [19]. DNA damage, which results in activation of the Hedgehog (Hh) signaling system, tends to result in BCC [108]. Overexpression of elements of Hh signaling has been reported in the epidermis and epidermal portion of hair follicles of adult  $VDR^{-/-}$  mice [109]. The Hh signaling, which involves Patched (Ptch), glioma-associated oncogene homolog (Gli1), and smoothened (Smo), has been shown to regulate cellular functions that are of importance for embryonic development and carcinogenesis [109]. It is now well accepted that mutations in the Ptch gene are a key event of BCC carcinogenesis [109]. Interestingly, it has been demonstrated that vitamin D compounds inhibit proliferation and growth of BCCs of Ptch mutant mice in vitro and in vivo via the VDR [109]. Several of the genes of the Hh pathway have sequences consistent with VDR response elements [33]. There is also consistent evidence that un-hydroxylated vitamin  $D_3$ , including the vitamin  $D_3$  produced as a result of UV exposure, inhibits Hh signaling in mouse skin [110], as well as UV-induced BCC in transgenic mice [111].

Higher baseline vitamin D status, assessed by blood concentrations of 25(OH)D, is mostly associated with higher incidence of skin cancers, BCC, SCC, and even in some cases of CMM [112] though some studies show an inverse [113] or no association [112]. There is, of course, considerable confounding due to sunlight exposure [114]. Whether keratinocyte and melanocyte cancers would be more prevalent without the protective effect of the vitamin D system in skin is unknown. One prospective study, which examined a single measurement of serum 25(OH)D in subjects in an Australian subtropical community and determined skin cancer risk over the subsequent 11 years, found no evidence that vitamin D status counteracted high sun exposure [115]. In the Women's Health Initiative Trial, those with a history of nonmelanoma skin cancer who were randomized to calcium (1000 mg) and vitamin D (400 IU/day) had a reduced risk of melanoma, though this was not seen in the whole cohort [116].

In contrast, lower 25(OH)D concentrations around the time of diagnosis in melanoma patients are associated with thicker tumors (higher Breslow thickness) [117,118], more ulceration and higher mitotic rate [119], and overall worse outcomes such as increased risk of progression and death over the following 5 years [112,117,120–122] and reviewed in Slominski et al., [123]. Whether supplemental vitamin D as adjuvant treatment would improve melanoma prognosis is unclear. One clinical trial was established as a pilot to examine safety of large initial vitamin D dose in melanoma patients at time of diagnosis with subsequent monthly doses [124] (Australia and New Zealand Clinical Trials Registry (ANZCTR) ACTRN12609000351213). Although the large and intermittent vitamin D dosing

may not have been ideal for a vitamin D effect [125,126], the trial found no safety issues, but was not powered for a clinical outcome (R. Saw—personal communication). A trial of adjuvant vitamin D supplementation (100,000 IU/50 days) after resection of a stage II melanoma found that Breslow thickness ( $< 3$  mm vs.  $> 3$  mm) was the primary determinant of disease-free survival with no significant effect of vitamin D supplementation, though 25(OH)D increased significantly less in patients with a Breslow score  $> 3$  mm [127]. Another clinical trial established to examine this question [128] (Clinical Trial.gov, NCT01748448, December 05, 2012) has not reported to date.

VDR (*Vdr*) knockout mice have been reported to develop increased skin tumors, mostly papillomas or BCC, after exposure to oral [129,130] or topical [131] DMBA. *Vdr* knockout mice are also more susceptible to tumor formation, papillomas, and increased SCC after chronic UV radiation [130]. Although there are many studies of associations between VDR polymorphisms and risk of keratinocyte cancers, there is little consistency between the various reports [21]. Polymorphisms in the *VDR* gene have been associated with risk or prognosis of BCC [132–134], SCC or its precursor, actinic keratoses [135,136], and melanoma [137–140] or nevi [141], though not all studies have shown significant associations.

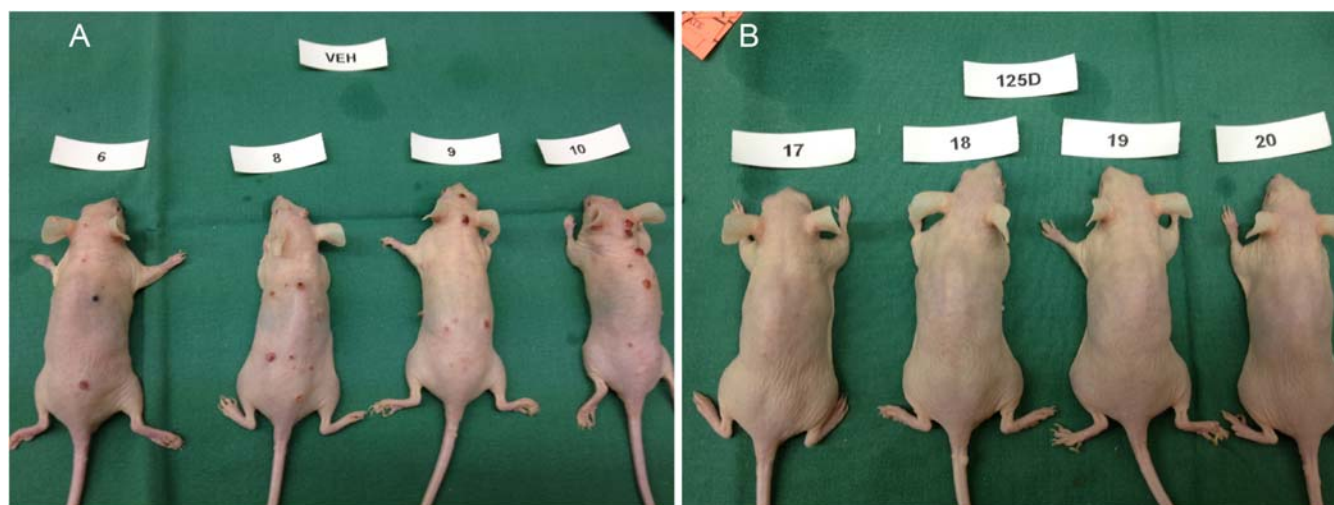
In relation to melanoma, though there is considerable inconsistency, overall, the *F* allele in *Fok1* is associated with a small (18%) increased risk for melanoma reviewed in Refs. [112,142], while the *B* allele of *Bsm1* is associated with a small (15%) decreased risk of melanoma, at least in some studies [143] and reviewed in Refs. [112,123,142]. Studies of *VDR* polymorphisms have produced highly variable results, but it seems that polymorphisms that are significantly associated with risk for melanoma mostly do not overlap with those related to melanoma survival [144] although other studies indicate some overlap [142]. Furthermore, the same polymorphisms such as rs73005032 can be associated with worse survival but lower melanoma risk [144]. VDR expression was lower in melanoma tumors than in normal skin and decreased with advanced tumor type [145]. Decreased nuclear VDR staining was associated with dermal invasion and metastasis [146]. In a study of over 3500 patients with melanoma, one functionally important VDR SNP—rs2239182, in the coding region of the *VDR* gene—was significantly associated with melanoma-specific death after adjustment for multiple testing [144]. Importantly, neither this SNP nor others found on primary analysis in that study were associated with Breslow thickness, ulceration, or mitosis [144]. VDR is induced in human skin with UVB exposure, at least at some doses, though not all [147], and in mouse skin and mouse skin explants [148].

Topical application of  $1,25(\text{OH})_2\text{D}_3$  inhibited chemically induced skin tumor formation [149]. Topical application of  $1,25(\text{OH})_2\text{D}_3$  also reduced skin tumors, including SCC, induced by chronic UV exposure [22] (Fig. 93.4). The effect of  $1,25(\text{OH})_2\text{D}_3$  was dose dependent. In that study, topical treatment with an analog with very limited capacity to promote transcription,  $1\alpha,25$ -dihydroxylumisterol [150], also reduced UV-induced skin tumors, including SCC, though the lumisterol derivative was less effective than  $1,25(\text{OH})_2\text{D}_3$  [22]. As noted, VDR knockout mice are more susceptible to chemically or UV-induced skin tumor formation [129,130]. On the other hand,  $1\alpha$ -hydroxylase (*Cyp27b1*) knockout mice, which cannot make  $1,25(\text{OH})_2\text{D}_3$  or, presumably, other  $1\alpha$ -hydroxylated metabolites, are not more susceptible to chemically induced skin tumors [129,151] or, reportedly, to UV-induced skin tumors [109]. *Cyp27b1* knockout mice have normal hair but impaired barrier function and wound healing [19]. These observations raise the possibility that other naturally produced metabolites, including CYP11A1-derived metabolites of vitamin D, such as  $20(\text{OH})\text{D}_3$  [99], may have photoprotective effects [21,123]. Some published studies showing reductions in UV-induced CPD and oxidative DNA damage in skin cells and mice with  $20(\text{OH})\text{D}_3$  support this proposal [152,153]. CYP11A1 metabolites of over-irradiation products such as lumisterol have now been described [102]. These compounds, rather than being biologically “inert,” could also contribute to photoprotection [154]. There are other “vitamin D-like compounds” which, although not based on cholesterol, potentially bind to the alternative binding pocket on the

VDR. These include curcumin and its metabolites [150,155]. Curcumin is a well-known anticancer agent, in part due to its antioxidant properties at micromolar concentrations [156]. Oral or topical application of curcumin or its major metabolite at high doses has been reported to reduce UV-induced skin tumor formation [23,157].

#### 4. Mechanisms of protection from UV-induced skin tumors

There are several mechanisms whereby vitamin D compounds and the VDR might protect from UV-induced skin tumors. These include protection from UV-induced DNA damage and enhanced repair, via several pathways including reductions in reactive oxygen and nitrogen species, which would result in less oxidative and nitritative DNA damage as well as enhanced repair due to less damage of repair-associated proteins. Other mechanisms include increased energy availability, alterations in nuclear p53 protein, and phosphorylation and effects on PTEN and related proteins. Vitamin D compounds and the VDR also modify the Hh pathway and  $\beta$ -catenin, important in BCC [19,109] and reviewed in Chapter 25, and alter long-noncoding RNA (lncRNA) profiles, changing the balance between oncogenic and tumor suppressor lncRNAs [18] and reviewed in Chapter 25. Immune responses to UV are modified by  $1,25(\text{OH})_2\text{D}_3$  and other vitamin D compounds [20,22]. Once tumors have developed, the anti-proliferative and pro-differentiation



**FIGURE 93.4** Skin tumors in mice after chronic UV exposure. Skh:hr1 hairless (but immune competent) mice were exposed to  $0.66 \text{ kJ/m}^2$  UVB and  $20.30 \text{ kJ/m}^2$  UVA, approximately one minimal erythral dose of solar-simulated UV, on 5 days per week for 10 weeks. Immediately after each exposure, the dorsum of each mouse was painted with either vehicle (ethanol: propylene glycol: water 2:1:1 v/v) or  $11.4 \text{ pmol/cm}^2$  of  $1,25(\text{OH})_2\text{D}_3$  in vehicle. The mice were monitored for a further 30 weeks [22]. Skin tumors on the dorsal surface of 4 mice treated with vehicle (A) and 4 mice treated with  $1,25(\text{OH})_2\text{D}_3$  are shown.

actions of  $1,25(\text{OH})_2\text{D}_3$  and related compounds are likely to contribute to observed better outcomes in patients with higher circulating  $25(\text{OH})\text{D}$  [14,120].

#### 4.1 Reduction in DNA damage and enhancement of repair

Numerous studies have shown that vitamin D metabolites and CYP11A1 metabolites of lumisterol reduce UV-induced DNA damage [22,55,154,158,159]. The hormone  $1,25(\text{OH})_2\text{D}_3$  can reduce CPD in mouse models, in Skh:hr1 hairless mice [22,160], C57BL/6 [130], and BALB/c mice [99], as well as in human keratinocyte, melanocyte, and fibroblast cells [54,158–160], human ex vivo skin [161], and human in vivo skin [162]. This effect is concentration dependent [22,54] and can be observed from 0.5 h after UV, the earliest time point tested [54,55,161]. Reductions in UV-induced DNA damage by vitamin D compounds have been demonstrated by immunohistochemistry and image analysis [158,159,163] as well as by Comet assay for short DNA strands [154] or after T4 endonuclease 5 (T4N5) digestion (where T4N5 cuts DNA at sites of CPD) or after Ogg1 endonuclease (which cuts DNA at sites of oxidative damage) followed by alkaline gel electrophoresis [55]. In keratinocytes, melanocytes, and fibroblast cultures and in mouse and human skin,  $1,25(\text{OH})_2\text{D}_3$  also protects cells from apoptosis following UV irradiation [22,54,158,159,162,164], probably due to less DNA damage when compared with vehicle-treated cells [54]. This may be related to the ability of  $1,25(\text{OH})_2\text{D}_3$  to initiate cell cycle arrest and reduce growth to facilitate repair [164].

UV-irradiated human and mouse skin treated with  $1,25(\text{OH})_2\text{D}_3$  exhibited significantly reduced 8-oxoguanine and 8-nitroguanine, compared with vehicle-treated skin [55,161]. Time course studies with Comet assays incorporating digestion with the Ogg1 repair enzyme show treatment with physiological doses of  $1,25(\text{OH})_2\text{D}_3$  immediately after UV exposure significantly reduced Ogg1-sensitive sites in irradiated keratinocytes from 0.5 h onward [55]. This has been further corroborated by nuclear staining with a monoclonal antibody to 8-oxoguanine [161]. 6-4 photoproducts after UV are also reduced by  $1,25(\text{OH})_2\text{D}_3$  and CYP11A1 derivatives of lumisterol [154].

Although it has been reported that preincubation with  $1,25(\text{OH})_2\text{D}_3$  for at least 8 h prior to UV is required for reduction in CPD after UV in human keratinocytes [158], these protective effects of vitamin D compounds have been observed in vitro and in vivo whether the agents were given before UV [54,162], or applied immediately after exposure to UV [22,152,161,165].

UV-produced CPDs are not reduced at a normal rate in *Vdr* knockout mice [130,148] or in mouse explants

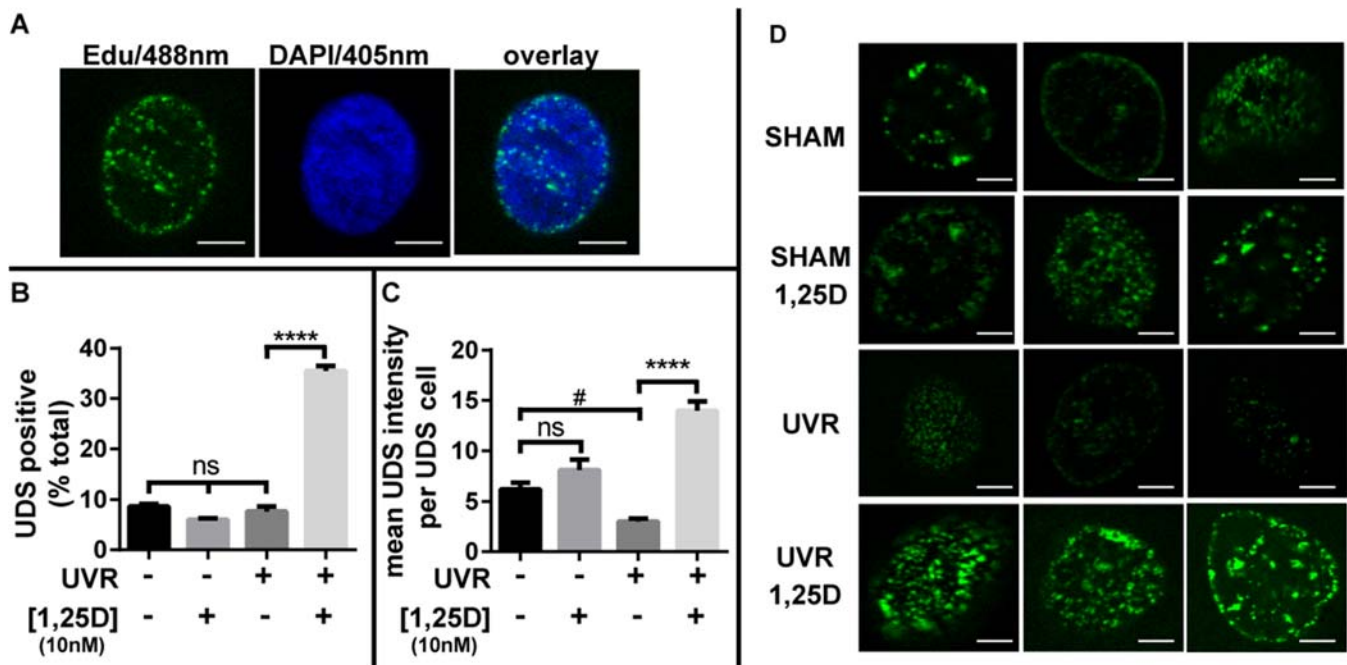
with reduced VDR [148] and neither CPD nor 8-oxoguanine was reduced in human keratinocytes in which either VDR or ERp57 had been knocked down [166]. As noted before, UV-generated CPD disappears at a much faster rate in a variety of model systems when  $1,25(\text{OH})_2\text{D}_3$  is provided [22,55,161]. These results may be reasonably interpreted to mean that DNA repair is reduced in *Vdr* knockdown conditions but enhanced by  $1,25(\text{OH})_2\text{D}_3$  and related compounds. There is now direct evidence of increased DNA repair in the presence of  $1,25(\text{OH})_2\text{D}_3$  demonstrated by increased unscheduled DNA synthesis in UV-exposed human keratinocytes (Fig. 93.5) [167].

#### 4.2 Role of VDR and other receptors

The VDR is clearly essential to the cancer prevention strategies of vitamin D steroids in skin. Zinser et al. first reported that VDR knockout mice were more susceptible to chemically induced skin tumors [129]. This was confirmed by Ellison et al. [130] who also reported that VDR knockout mice were far more susceptible to UV-induced skin tumors than wild-type mice. This has been confirmed by others [109].

As discussed in Chapters 10–13, most actions of the vitamin D hormone are mediated by the classical genomic pathway that results mainly in modulation of gene transcription [168]. There is reasonable evidence for the existence of an alternate, non-classical pathway that appears to be important for some actions of  $1,25(\text{OH})_2\text{D}_3$  [169,170], including photo-protection. This non-classical pathway is discussed in more detail in Chapter 22. The chemically synthesized, *cis*-locked  $1\alpha,25$ -dihydroxylumisterol<sub>3</sub> has little capacity to alter transcription of vitamin D target genes and so is considered an agonist of the non-classical pathway [150,171].  $1\alpha,25$ -dihydroxylumisterol<sub>3</sub> and other naturally occurring CYP11A1 lumisterol derivatives, which also have little trans-activating activity, reduced UV-generated CPD and other types of DNA damage to a similar extent as  $1,25(\text{OH})_2\text{D}_3$  in human primary melanocytes, fibroblasts, and keratinocytes [22,159,160,165].  $1\beta,25$ -Dihydroxyvitamin D<sub>3</sub> (HL) is an antagonist of the non-classical pathway [150], while (23S)-25-dehydro- $1\alpha$ -OH-D<sub>3</sub>-26,23-lactone (MK/TEI) is an antagonist of the classical vitamin D pathway [172]. Studies in all these cell types have clearly demonstrated that neither  $1\beta,25$ -dihydroxyvitamin D<sub>3</sub> (HL) nor (23S)-25-dehydro- $1\alpha$ -OH-D<sub>3</sub>-26,23-lactone (TEI) alters CPD after UV on their own. The classical antagonist, (23S)-25-dehydro- $1\alpha$ -OH-D<sub>3</sub>-26,23-lactone (TEI), had no effect on photo-protection by  $1,25(\text{OH})_2\text{D}_3$  in this system, but the presence of the non-classical antagonist  $1\beta,25$ -dihydroxyvitamin D<sub>3</sub> (HL) completely abolished the





**FIGURE 93.5** Unscheduled DNA synthesis was increased in primary human keratinocytes after treatment with  $1,25(\text{OH})_2\text{D}_3$  following UV irradiation. (A) Confocal microscopy image of the nucleus of an ssUV-irradiated keratinocyte scanned for thymidine analog (EDU) incorporation at 488 nm and counterstained with DAPI. This punctate staining of nuclear EDU was considered positive for unscheduled DNA synthesis (UDS). Scale bar represents 5  $\mu\text{m}$ . (B) Percentage of UDS-positive cells (as shown in A) as a proportion of total cells randomly counted by DAPI counterstain from keratinocytes 90 mins following UV irradiation (UVR), or nonirradiation, in response to 10 nM  $1,25(\text{OH})_2\text{D}_3$  or the vehicle treatment (-).  $n = 1000$  cells counted per treatment between triplicate experiments. (mean  $\pm$  SEM). Significantly different from all other treatment groups  $****P < .0001$ ; ns = no sig. diff. (C) Average intensity of EDU incorporation per UDS-positive cell, as measured by densitometry,  $n = 36$  UDS-positive cells quantified per treatment between triplicate experiments. (mean  $\pm$  SEM). Significantly different from UVR without  $1,25(\text{OH})_2\text{D}_3$   $****P < .001$ ; significantly different from vehicle treated nonirradiated  $\#P < .05$ . (D) Example images of cells used for the EDU intensity analysis shown in C (SHAM cells = nonirradiated, i.e., shielded from irradiation). Scale bar represents 5  $\mu\text{m}$ . Reproduced from Ref. [167] with permission.

reductions in CPD due to the presence of  $1,25(\text{OH})_2\text{D}_3$  [22,160,165].

As noted earlier, mice in which VDR has been deleted are more susceptible to UV-induced skin tumors [130]. VDR knockdown in human keratinocytes also abolished the reductions in CPD and 8-oxoguanine damage after UV with  $1,25(\text{OH})_2\text{D}_3$  as well as  $20(\text{OH})\text{D}_3$  and the lumisterol derivative,  $24(\text{OH})\text{L}_3$  [166]. The requirement for VDR was also examined in fibroblasts from patients with hereditary vitamin D-resistant rickets who have mutations in VDR [173]. Addition of  $1,25(\text{OH})_2\text{D}_3$  did not reduce CPD after UV in fibroblasts from a patient with a mutation resulting in an early stop codon in the VDR gene (R50X), which expressed no detectable VDR (VDR null fibroblasts) [173,174]. On the other hand, fibroblasts from a patient with a missense mutation in the region coding for the DNA-binding domain (V26M) [175] (DBD-mutant fibroblasts) still showed significant reductions in UV-induced CPD after addition of  $1,25(\text{OH})_2\text{D}_3$  or the cis-locked compound,  $1\alpha,25$ -dihydroxylumisterol $_3$ . This indicates that at least reductions in post-UV CPD could be mediated by a VDR with little ability to bind to DNA or to induce genomic

transactivation. Further studies with fibroblasts from a patient with a 5-base-pair deletion/8-base-pair insertion in Helix 1 of the VDR ligand-binding domain (LBD), which abolished classical ligand binding [176], still allowed  $1,25(\text{OH})_2\text{D}_3$  or  $1\alpha,25$ -dihydroxylumisterol $_3$  to reduce post-UV CPD [173]. The interpretation of these results is that a VDR is needed for reductions in UV-induced CPD by vitamin D compounds, but that receptor needs not be able to bind to DNA or exhibit classical binding to  $1,25(\text{OH})_2\text{D}_3$ .

What about ERp57/PDIA3/MARRS, which has been proposed to be the main binding protein for  $1,25(\text{OH})_2\text{D}_3$  and related compounds for nonclassical responses [177] or at least a critical element in mediating responses such as rapid induction of intestinal calcium transport [170,178]? When ERp57 was knocked down in normal human fibroblasts or keratinocytes by siRNA,  $1,25(\text{OH})_2\text{D}_3$  failed to reduce post-UV CPD [173]. Likewise, the use of a neutralizing antibody to ERp57 (Ab 099, a rabbit polyclonal antibody that recognizes the N-terminal of ERp57 [177] and that could only access a cell membrane associated protein) abolished the protective effect of  $1,25(\text{OH})_2\text{D}_3$  in skin fibroblasts [173]. In this



context, it is worth noting that the classical VDR has been found to be present on the cell membrane in association with caveolae [179]. Furthermore, in normal skin fibroblasts, VDR and ERp57 were coimmunoprecipitated in nonnuclear cellular lysates [173]. Similarly, protective effects of  $1,25(\text{OH})_2\text{D}_3$  and CYP11A1 derivatives of vitamin  $\text{D}_3$ , such as  $20(\text{OH})\text{D}_3$  or of lumisterol, such as  $24(\text{OH})\text{L}_3$ , against both CPD and oxidative DNA damage, were abolished in normal human keratinocytes when either the VDR or ERp57 was knocked down by siRNA [166].

As noted in Chapter 22, in chondrocytes and other cell types, the effects of  $1,25(\text{OH})_2\text{D}_3$  and other agents such as  $1\alpha,25$ -dihydroxylumisterol $_3$  and  $25(\text{OH})\text{D}_3$  on ion channel currents via nonclassical pathways involve the opening of chloride channels [150,171,180]. Chloride channel opening is inhibited by 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), and in the presence of DIDS, this nonclassical pathway is blocked [150,171,180]. In human keratinocytes, DIDS had no effect on its own, but in its presence, there was no reduction in CPD after UV with  $1,25(\text{OH})_2\text{D}_3$  [181].

Not all actions of  $1,25(\text{OH})_2\text{D}_3$  or related compounds in skin cells that are potentially useful in photoprotection require the VDR. These actions include the increase in overall p53 expression [173], inhibition of Smoothed, part of the Hh pathway [110,182]. Not all actions are inhibited by DIDS, such as reductions in post-UV nitrotyrosine [181]. Nevertheless, overall, the data are consistent with the hypothesis that, at least in part, vitamin D compounds bind to the alternative pocket on the VDR at the cell membrane, which in turn, through an action that involves the protein ERp57 at the cell membrane, results in opening of chloride channels, and this leads to several functional changes in UV-irradiated cells, which facilitate reductions in CPD.

DNA repair involves substantial chromatin remodeling [57], and  $1,25(\text{OH})_2\text{D}_3$  regulates epigenetic mechanisms that maintain the transcription of its target genes that contribute to its regulatory network [183,184]. Though direct interaction of the liganded VDR with chromatin remodeling proteins has been recently described [185], much VDR-mediated chromatin remodeling occurs via classic genomic pathways. For example, the VDR/RXR dimer recruits histone acetyltransferases (HATs) such as p300/CBP and steroid receptor coactivators 1 and 2 (SRC-1 and SRC-2), which carry out the acetylation reactions that open up the chromatin structure to facilitate transcription [168]. Other photoprotective mechanisms, such as suppression of Hh pathway signaling, and induction of metallothionein and XPC mRNA, appear to be via classic genomic alterations in gene transcription [186]. It has been proposed

that some actions of vitamin D- or lumisterol-derived compounds may be mediated by retinoic acid orphan receptors ROR $\alpha$  and  $\gamma$  or the aryl hydrocarbon receptor since some of these compounds such as  $20\text{S}(\text{OH})\text{D}_3$  and  $20,23(\text{OH})_2\text{D}_3$  have been shown to act via these receptors [154].

As indicated before, agents such as  $1\alpha,25$ -dihydroxylumisterol $_3$  and  $20(\text{OH})\text{D}_3$ , which have been characterized as agonists of the nonclassical pathway with little transactivating activity, also reduce DNA damage and UV-induced immune suppression in mice [22,152]. Since DNA damage and immune suppression both contribute to photo-carcinogenesis, it is likely that nonclassical pathways play an important role in protection from UV-induced tumors. It should be noted, however, that although  $1\alpha,25$ -dihydroxylumisterol $_3$  appeared equivalent to  $1,25(\text{OH})_2\text{D}_3$  in reducing both UV-induced DNA damage and immune suppression in mice, it was less effective than  $1,25(\text{OH})_2\text{D}_3$  in reducing UV-induced skin tumors or SCC in these animals [22], suggesting that at least some of the photoprotective effects of  $1,25(\text{OH})_2\text{D}_3$  are due to genomic mechanisms. Another low calcemic vitamin D analog, which has some transactivating capacity, 1-hydroxymethyl-16-ene-24,24-difluoro-25-hydroxy-26,27-bis-homovitamin  $\text{D}_3$ , QW-1624F2-2 (QW), which has little calcemic effect [187], reduced levels of UV-induced CPD in primary human keratinocytes, dermal fibroblasts [188], melanocytes, and in mouse skin in vivo [160] as well as inhibiting UV-induced immune suppression [160,188]. In these studies, its photoprotective effectiveness was equivalent to that of  $1,25(\text{OH})_2\text{D}_3$  [160]. QW reduced acute UV-induced DNA and immune system damage and at a dose 10-fold that used in UV studies reduced skin tumors in a model of chemical-induced skin carcinogenesis [189]. At the single dose tested in a chronic UV protocol, however, QW was not effective to reduce UV-induced skin carcinogenesis [188]. A similar failure to reduce UV-induced photo-carcinogenesis also was reported for some other vitamin D-derived and lumisterol-derived agents [23,24,26]. In contrast, the vitamin D-like agent curcumin reduced UV-induced tumors whether given orally or topically [157], and its major metabolite, tetrahydrocurcumin, effectively reduced UV-induced tumors after topical application [23]. While there may be other markers of efficacy to reduce UV-induced skin tumors (see "other potential contributors to photo-carcinogenesis" in the following), constraints of photo-carcinogenesis studies include the length of the studies (40 weeks) and the large number of mice needed per treatment group ( $n = 20$ ), which make them labor-intensive and expensive, thus limiting the number of treatment doses that can be tested in a single study.

### 4.3 Reduced generation of reactive oxygen and nitrogen species

During normal cellular metabolism, the continually generated low levels of ROS play a role in many normal cellular processes including growth control and differentiation [190]. In addition, activation of photoreceptors in skin by UV absorption generates free radicals by electron transfer or hydrogen abstraction processes in other molecules, or by energy transfer to molecular oxygen increasing the rate of ROS production [191]. Redox balance is regulated by intrinsic cellular antioxidant systems, which consist of both antioxidant enzymes and non-enzymatic antioxidants [192]. For example, superoxide anions are converted by superoxide dismutase to hydrogen peroxide, which is converted to water and oxygen by catalase. Insufficient enzyme activity increases the levels of superoxide and hydrogen peroxide that form highly toxic peroxynitrite and hydroxyl ions, respectively. Non-enzymatic free radical scavenging antioxidants, glutathione, metallothionein, thioredoxin, vitamins C and E, and carotenoids are also present in skin, but may be inactivated by UV-induced ROS and RNS. Glutathione peroxidase, superoxide dismutase, and catalase expression are downregulated for several days following UV exposure, which would permit an exponential increase in ROS and RNS [193]. Inflammatory cells induced by UV also migrate into irradiated skin and may contribute to increased levels of ROS [88].

UV-induced ROS can overcome the intrinsic enzymatic and non-enzymatic antioxidant systems within the skin, causing an imbalance between ROS and antioxidant systems. Loss of cellular redox homeostasis from UV has been implicated in both photo-carcinogenesis and photo-aging. The resulting oxidative stress would perturb cellular defenses against DNA damage, oxidative and nitrative modifications to proteins, and lipid peroxidation of membranes, leading to cell death.

UV radiation is a very potent oxidative stressor leading to significant alterations in the activity of the nuclear factor-erythroid-2-related factor 2 (Nrf2). The activity of the redox-sensitive transcription factor Nrf2 is regulated by a number of mechanisms, which determine its nuclear import/export balance and its degradation [194]. Nrf2 activates genes that encode antioxidant enzymes such as catalase, superoxide dismutase, and others [195]. In the absence of oxidative stress, Nrf2 binds to Kelch-like ECH-associated protein (Keap 1). Keap 1 links Nrf2 to the cytoskeleton to retain Nrf2 in the cytoplasm, thereby promoting its degradation [194]. On the other hand, oxidative stress enables Nrf2 to escape Keap1-mediated proteasomal degradation, leading subsequently to Nrf2 stabilization and its translocation into the nucleus [194]. There is evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub> activates the Nrf2–Keap1 antioxidant pathway which

ameliorates oxidant stress [196–198] including in UV-exposed keratinocytes [154] and thus potentially, oxidative DNA damage.

Treatment of keratinocytes with 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited the activation of stress-activated protein kinases, such as c-Jun N-terminal kinase (JNK), [199] and inhibited apoptosis induced by oxidative stress, TNF $\alpha$  and hydrogen peroxide cytotoxicity [200–202], possibly by increasing inherent antioxidant systems [154]. Overproduction of NO can alter the mitochondrial membrane potential. This can allow the release of pro-apoptotic proteins, activating the mitochondrial apoptotic pathway [203]. There is some evidence that the VDR acts as a gatekeeper of mitochondrial respiratory chain activity. In immortalized keratinocytes, HaCaT cells, VDR silencing increased transcription of the subunits II and IV of cytochrome c oxidase and was accompanied by sharp increases in the mitochondrial membrane potential, which sensitized the cells to oxidative stress [204]. There is also some evidence that mitochondrial damage after UV is repaired more quickly in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> [167]. Consistent with UV-induced mitochondrial damage [205], the mitochondrial protein PINK1 was increased in human keratinocytes after a dose of UV that was not high enough to increase caspase activity [167]. This was accompanied by an increase in ROS [167]. In this study [167], when 1,25(OH)<sub>2</sub>D<sub>3</sub> was added to keratinocyte cultures immediately after UV exposure, there was evidence of enhanced mitochondrial-derived vesicle-dependent mitophagy and suppression of mitochondrial membrane potential, both associated with decreased ROS [206,207].

Metallothionein is a cysteine-rich protein, which functions in metal detoxification and is a potent oxygen radical scavenger. When metallothionein was upregulated by cadmium in UV-irradiated mouse skin, the result was reduced UV-induced apoptotic sunburn cells and reduced photodamage [208]. Superoxide and hydroxyl radicals were also reduced. Induction of metallothionein by 1,25(OH)<sub>2</sub>D<sub>3</sub> was first observed in mouse keratinocytes in vitro as well as mouse liver, kidney, and skin in vivo by Karasawa and colleagues [209]. Further studies demonstrated an inhibition of UV-induced keratinocyte death in vitro and a reduction in sunburn cells in vivo in mouse skin following 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment, with a concurrent increase in metallothionein levels, though independently of glutathione [163,210]. There is also a report that 1,25(OH)<sub>2</sub>D<sub>3</sub> increased glutathione and reduced ROS in monocytes through upregulated glutathione glutamate cysteine ligase and glutathione reductase, which catalyze glutathione biosynthesis [211]. There is evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs can directly induce antioxidant genes, for example, glucose-6-phosphate

dehydrogenase, glutathione peroxidase, and thioredoxin reductase [212]. It was shown that  $1,25(\text{OH})_2\text{D}_3$  protected against hydrogen peroxide-induced cell death in prostate epithelial cell lines through transcriptional activation of glucose-6-phosphate dehydrogenase, followed by an increase in glutathione levels [212]. A protective effect of  $1,25(\text{OH})_2\text{D}_3$  in keratinocytes against not only UV but also treatment with  $\text{TNF}\alpha$  or ceramides has been reported. This appeared to be mediated by production of sphingosine-1 phosphate, which prevented apoptosis [213]. Relatively high doses of  $1,25(\text{OH})_2\text{D}_3$  (approximately  $10^{-7}$  M), however, were required to provide protection in this study [213].

Vitamin D compounds are not antioxidants, and there is even evidence that  $1,25(\text{OH})_2\text{D}_3$  increases reactive oxygen species in adipose tissue [214]. In skin, however, there is ample evidence that  $1,25(\text{OH})_2\text{D}_3$  and related compounds reduce both reactive oxygen and nitrogen species after UV [22,54,154,167]. The decrease in nitrite in keratinocyte cultures with  $1,25(\text{OH})_2\text{D}_3$  was similar to that seen with nitric oxide synthase inhibitors, aminoguanidine and L-N-monomethylarginine [54]. Comparable reductions were also shown by whole-cell ELISA specific to detect 3-nitrotyrosine [22,181]. As noted earlier, the mechanisms for reductions in ROS and RNS appear to include increased Nrf2 as well as its targets heme oxygenase-1, catalase, and superoxide dismutase/Mn [154] together with enhanced repair of mitochondrial damage [167].

Reduction in reactive oxygen or nitrogen species generated would directly reduce the number of oxidized or nitrated guanine bases, as reported [55,161]. Reduced nitric oxide intermediates would also facilitate DNA repair, since there is evidence that nitric oxide and its intermediates inhibit repair of CPD by nitrosylation of DNA repair proteins and preferential inhibition of excision and ligation steps of nucleotide excision repair [62,215]. Furthermore, peroxynitrite, produced as a result of a chemical reaction between nitric oxide and superoxide, has been reported to inactivate Ogg1, a key base excision repair enzyme, critical for repairing both 8-oxoguanine and 8-nitroguanine [61]. These results are consistent with the proposal that the action of  $1,25(\text{OH})_2\text{D}_3$  to reduce DNA damage after UV irradiation may be partly due to a decrease in ROS and RNS, which leads to reduced oxidative and nitrative DNA damage as well as increased repair of all types of DNA damage [22,54,216].

#### 4.4 Role of tumor suppressor protein p53

DNA damage, such as after UV exposure, activates the tumor suppressor p53, a 53 kDa protein involved

in regulation of the cell cycle and facilitation of DNA repair [38,217]. After DNA damage, p53 is modified by phosphorylation and acetylation of at least 20 sites through different stress signaling pathways, leading to accumulation of p53 in the nucleus [217]. Under normal physiological conditions, p53 is present in cells at very low levels due to its very short half-life of less than 30 min [218,219]. In normal cells, p53 is bound to its negative regulator, the MDM2 protein. MDM2 maintains p53 at low levels by increasing its susceptibility to proteolysis by the 26S proteasome. The MDM2 gene is a proto-oncogene, which functions mainly to modulate the activity of p53 [219]. Modifications in p53 enhance its accumulation mostly by inhibiting degradation facilitated by MDM2. These processes lead to nuclear accumulation of p53 that reaches maximum levels 12 h after UV radiation [218]. While phosphorylation of p53 at Ser<sup>46</sup> is important for apoptosis control [220], phosphorylation at Ser<sup>15</sup> may be important for accumulation of p53 in the nucleus and for transactivation of downstream genes to induce cell cycle arrest in the G1 phase, and other actions that facilitate DNA repair [221]. If the DNA damage is too severe to be repaired, apoptotic pathways are activated to eliminate the damaged cell before it replicates. p53 can upregulate the expression of pro-apoptotic genes such as Bax and Fas/Apo-1, or can downregulate expression of anti-apoptotic genes such as Bcl-2 [43]. In addition to disrupting the cell cycle to allow time for DNA repair, p53 directly affects the transcription of genes that control NER [222,223] and base excision repair [66] pathways, as well as pro- or anti-apoptotic pathways [43]. p53 mediates the transcription of GADD45, which assists DNA repair by binding to DNA, increasing its accessibility to repair enzymes [224].

The p53 tumor suppressor gene is one of the most commonly mutated genes in UV-induced skin cancers [43,225]. Physiological doses of UVA and UVB can induce inactivating mutations in p53. Mutations in the tumor suppressor p53 in engineered human skin were found to be predominantly UVA finger print mutations induced by oxidative damage located in the basal layer of the epidermis [38]. Under circumstances where there are mutations in p53, reduced global genome repair of CPD is observed, but transcription-coupled repair continues [226]. A positive association between mutations in the tumor suppressor p53 gene in UV damaged skin in mouse and human skin before skin tumors appear provides evidence for their involvement in skin carcinogenesis [38]. DNA repair was blocked in cells transfected with a dominant-negative p53 [227], and early-onset tumor formation increased in homozygous p53 knockout mice [228]. The gene for the DNA strand sensor protein kinase (ATM) acts by phosphorylating p53 at serine 15 and is inactivated in patients with the genetic disorder ataxia telangiectasia. In this disease,



patients suffer from genome instability and cancer [229]. Several p53 phosphorylation sites are commonly inactivated as a result of UV-induced DNA damage. When such mutations are engineered in mice, they promote photo-carcinogenesis [230].

Increased nuclear p53 protein expression, beyond the increase with UV alone, has been reported by 3 h post-exposure in human keratinocytes and melanocytes treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> [54,160], though not in all studies [153]. Increased phosphorylation of p53 at Ser<sup>15</sup> occurs after UV [167], with nuclear accumulation of p53-Ser<sup>15</sup> [154]. While addition of CYP11A1 derivatives of vitamin D<sub>3</sub> or lumisterol leads to further increases in nuclear accumulation of p53-Ser<sup>15</sup> [154], treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> itself does not further increase p53-Ser<sup>15</sup> [167] or its nuclear accumulation [154]. In human skin fibroblasts that did not express the VDR, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment increased total cellular p53 expression, but did not protect against UV-induced CPD [173]. Thus, while further upregulation of p53 by 1,25(OH)<sub>2</sub>D<sub>3</sub> after UV, possibly by a VDR independent process [130,173], would be likely to contribute to enhanced DNA repair, the role that this process plays in reducing post-UV DNA damage in the presence of vitamin D compounds is currently unclear.

#### 4.5 DNA repair proteins and processes

There is some evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub> may enhance expression of proteins involved in DNA damage recognition or repair. Moll et al., using array technology, reported that treatment of human keratinocytes in culture with 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulated mRNA for the CPD damage recognition proteins DDB2 (part of the XPE complex) and XPC [231]. Song reported significantly increased expression of DDB2 and XPC protein in biopsies of human subjects exposed to UV and treated topically with 1,25(OH)<sub>2</sub>D<sub>3</sub> or a vitamin D-like compound [216], which was confirmed in human skin explants [232]. It is possible that this 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced increase in XPC is a consequence of p53 upregulation [233]. Evidence of the importance of this upregulation of XPC by 1,25(OH)<sub>2</sub>D<sub>3</sub> after UV is that knockdown of XPC in human keratinocytes with siRNA to XPC, compared with control siRNA, abolished the reduction in CPD in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> [234].

There may also be important effects of VDR on epigenetic actions of DNA damage recognition proteins. There is evidence that in keratinocytes from VDR knockout mice, the key DNA repair protein XPC accumulated normally at sites of DNA damage but dissociated far more slowly compared with control keratinocytes [32]. The slower dissociation of XPC led to decreased binding of XPF endonuclease, which

reduced the rate of removal of DNA damage in the form of pyrimidine–pyrimidone (6–4) photoproducts in VDR-depleted skin cells [32].

The mechanism of increased XPC expression after UV and 1,25(OH)<sub>2</sub>D<sub>3</sub> may involve phosphatase and tensin homolog deleted on chromosome 10 (PTEN). PTEN is a negative regulator of the oncogenic acutely transforming retrovirus AKT8 in rodent T cell lymphoma (AKT) pathway and acts as a tumor suppressor. PTEN is defective in a number of different cancers, including glial, prostate, breast, lung, endometrial cancers, and melanoma [235]. Susceptibility to tumor induction was increased in mice with PTEN deletion, and PTEN levels were lower in SCC and papillomas compared with normal skin in a mouse model of UV-induced skin carcinogenesis [236]. Furthermore, PTEN levels were reduced in SCC and actinic keratoses from human subjects compared with normal skin [237]. Activation of the AKT pathway allows for evasion of cell death pathways, providing favorable conditions for cancer progression. AKT pathway activation commences with the phosphoinositide 3-kinase (PI3K)-induced conversion of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to phosphatidylinositol-3,4,5-trisphosphate (PIP<sub>3</sub>) [238]. Conversely, PTEN inhibits PI3K-dependent signaling by dephosphorylating PIP<sub>3</sub> to PIP<sub>2</sub>. PTEN thus decreases levels of activated AKT and enables effective cell cycle regulation.

Studies by Ming and colleagues showed that UVB downregulates PTEN in the immortalized keratinocyte line, HaCaT, as well as in primary human keratinocytes and in mouse skin in an AKT and extracellular signal-regulated kinase (ERK)<sub>1/2</sub>-dependent manner [237]. Further studies by this group showed that PTEN downregulation impairs global genomic NER, which is necessary for removal of UV-induced DNA lesions including CPD. Specifically, loss of PTEN resulted in suppression of a key player in the global genomic NER process, the xeroderma pigmentosum group C protein (XPC), which contributes to global genomic NER by way of DNA damage recognition. This was shown to be mediated through AKT and p38 signaling [239]. Studies by Shariev et al. demonstrated that solar-simulated UV decreased PTEN levels in primary human melanocytes and in Skh:hr1 hairless mouse skin, as well as in human melanoma cells [240]. Addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited this UV-induced depletion of PTEN [240] likely in part due to suppression of AKT phosphorylation after UV [167]. Taken together with the study noted before by Song et al. showing 1,25(OH)<sub>2</sub>D<sub>3</sub> increased XPC protein expression in biopsies of UV-exposed human skin [216], PTEN is a likely target for 1,25(OH)<sub>2</sub>D<sub>3</sub> in its protective effect against UV-induced DNA lesions. Indeed, it has been suggested that the VDR can bind the PTEN promoter in gastric cancer cells to inhibit apoptosis [241].



PTEN may also be important at later stages of carcinogenesis in that PTEN contributes to melanoma development [242] and  $1,25(\text{OH})_2\text{D}_3$  has been shown to reduce melanoma cell viability in a PTEN-dependent process [243].

DNA repair is a process requiring *energy*, but energy generation by skin cells is greatly reduced after UV exposure through several mechanisms [244,245]. There is decreased ATP production from oxidative phosphorylation due to damage to mitochondria [246]. UV irradiation of keratinocytes increases poly(ADP-ribose) polymerases (PARPs), principally PARP-1, which also results in energy depletion [245].

As noted before, addition of  $1,25(\text{OH})_2\text{D}_3$  to UV-exposed keratinocytes enhances the mitochondrial repair process of mitophagy. This reduces ROS but does not increase oxidative phosphorylation measured by oxygen consumption rates on SeaHorse analysis [166,167]. Furthermore, use of the oxidative phosphorylation inhibitor oligomycin does not alter CPD reductions in the presence of  $1,25(\text{OH})_2\text{D}_3$  [166,167].

In contrast, the glycolysis inhibitor, 2-deoxyglucose, when added to UV-irradiated keratinocytes, completely blocks the reduction in CPD in the presence of  $1,25(\text{OH})_2\text{D}_3$ , indicating that glycolysis is the major source of energy for DNA repair after UV [167]. This increased glycolysis is likely to involve suppression of PARP-1 activity. After UV radiation, formation of DNA strand breaks activates pathways that consume nicotinamide adenine dinucleotides (NAD). PARPs are principal agents in this process, which function to transform NAD into polymers of ADP-ribose and thus modify proteins post-translation [247]. PARP-1, the oldest member of the PARP family, is induced by UV irradiation and is typically involved in DNA repair and cell death and has been shown to modulate chromatin remodeling, an essential step in DNA repair [248].

As noted before, exposure to UV results in induction of PARP-1, which is directly activated upon recognition and binding to damaged DNA [245]. It has previously been thought that PARP-1-induced cell death arose from energy depletion mainly due to excessive use of NAD<sup>+</sup> within the cell [245]. Certainly, replacement with oral nicotinamide has been shown to reduce new non-melanoma skin cancers and actinic keratoses in human subject over 12 months in a phase III clinical trial [249]. Perhaps more importantly, PARP-I inhibits hexokinase, the rate-limiting enzyme in glycolysis [250,251]. It seems that  $1,25(\text{OH})_2\text{D}_3$  is able to suppress PARP-1 activity in immortalized keratinocytes [252] and normal human keratinocytes [26], which would alleviate the blockade of glycolysis. UV stimulates phosphorylation of ERK<sub>1/2</sub> [239]. Increased ERK<sub>1/2</sub> phosphorylation has been linked to PARP-1 activation [253], and  $1,25(\text{OH})_2\text{D}_3$  inhibits UV-induced ERK<sub>1/2</sub>

phosphorylation, a potential explanation for suppression of PARP-1 [167].

As well as increased ERK<sub>1/2</sub> phosphorylation, UV exposure stimulates phosphorylation of AKT [239]. These, in turn, activate mammalian target of rapamycin complex 1 (mTORC1) to promote growth and inhibit autophagy [19,167,254]. It should be noted that alterations in phosphokinase signaling by  $1,25(\text{OH})_2\text{D}_3$  after exposure to UV are dual in nature. While phosphorylation of AKT, ERK, mTOR, and others that support energy-costly growth pathways were suppressed with  $1,25(\text{OH})_2\text{D}_3$ , pathways that are important for DNA repair and cell survival, such as phosphorylation of p53, stress-activated protein kinase/cJun N-terminal kinase, or UV resistance-associated gene (UVRAG), were maintained at the increased levels seen after UV exposure [167].

One pathway for energy conservation is autophagy [255], a process that is reportedly enhanced by  $1,25(\text{OH})_2\text{D}_3$  in monocytes/macrophages as well as breast cancer cells [256–258] and probably in keratinocytes [259]. Treatment of keratinocytes with  $1,25(\text{OH})_2\text{D}_3$  immediately after solar-simulated UV (ssUV) irradiation increased autophagy through alterations in several signaling pathways including suppressed phosphorylation of ERK<sub>1/2</sub>-Thr<sup>202</sup>Y<sup>204</sup>, AKT-Ser<sup>473</sup>, and mTOR Ser<sup>2448</sup> acting through glycogen synthase kinase-3 (GSK<sub>3</sub>) [167]. Although increased autophagy was not essential for the observed increases in repair of DNA damage [167], changes in these signaling pathways have many functional effects contributing to enhanced DNA repair [19,167].

#### 4.6 Vitamin D compounds and UV-induced immune suppression

As noted before, UV radiation is an important environmental immune suppressant in humans [88] and that suppression is key to skin tumor development [260,261]. Topical application of  $1,25(\text{OH})_2\text{D}_3$  to non-UV-exposed human skin has been found to induce immune suppression at a similar level to that resulting from UV irradiation only [162]. Application of the  $1,25(\text{OH})_2\text{D}_3$  analog, calcipotriol, also caused immune suppression in people and mice [262,263] but also suppressed polymorphic light eruptions, also known as “sun allergy” [264,265].

In other studies in mice, topical application of  $1,25(\text{OH})_2\text{D}_3$  and related compounds, reduced UV-induced systemic immunosuppression [22,23,26,152,266]. The vitamin D-induced protection against photo-immune suppression has been shown to display a gender bias, with greater protective effects observed in female compared with male mice [266]. Male mice and human males are

more susceptible to UV-induced immune suppression [90,91]. Male mice are more immune suppressed by topical  $1,25(\text{OH})_2\text{D}_3$  at lower doses than female mice [266]. Reduced susceptibility of female mice to immune suppression may indicate a link between female sex hormones/receptors and  $1,25(\text{OH})_2\text{D}_3$  actions.  $1,25(\text{OH})_2\text{D}_3$  has been reported to modulate estrogen receptor expression [267,268]. The estrogen receptor  $\beta$  is the only estrogen receptor in mouse skin [269]. In female transgenic mice that did not express estrogen receptor  $\beta$ , protection by  $1,25(\text{OH})_2\text{D}_3$  against photo-immune suppression was not demonstrated [266]. Use of an estrogen receptor  $\beta$  antagonist had a similar effect [266], suggesting that the presence of a functional estrogen receptor  $\beta$  in skin contributes to reduced UV immunosuppression and to detection of a protective effect by  $1,25(\text{OH})_2\text{D}_3$ .

The protection by  $1,25(\text{OH})_2\text{D}_3$  from photo-immune suppression whether assessed by contact hypersensitivity response or by delayed-type hypersensitivity response has also been observed in C3H and C57Bl/6 female mice [270]. In a study by a different group, vitamin D-replete status protected C57Bl/6 mice from UV-induced immune suppression, as well as DNA damage, but was not effective in BALB/c mice [271]. It is not yet clear why the use of different model systems produces different results, although, apart from sex and species differences, different UV sources, differences in the doses of UV and the spectrum delivered and different doses of topical  $1,25(\text{OH})_2\text{D}_3$  may contribute.

Another proposed mechanism for the observed  $1,25(\text{OH})_2\text{D}_3$ -mediated reduction of post-UV immune suppression in some models is a reduction in the expression of the inflammatory cytokine, IL-6 [79,199], which is itself a potent inducer of IL-10, an endogenous immunosuppressant [78]. Furthermore, as noted earlier, metallothionein mRNA expression is induced by  $1,25(\text{OH})_2\text{D}_3$  in cultured epidermal keratinocytes [209]. Since UV-induced immune suppression was increased in metallothionein knockout transgenic mice [272,273], this mechanism might be responsible, at least in part, for the reduction in photo-immune suppression by  $1,25(\text{OH})_2\text{D}_3$ .

UV exposure increases pro-inflammatory cytokines such as IL-6 and tumor necrosis factor  $\alpha$  probably via activation of the nuclear factor kappa B (NF- $\kappa$ B) signaling pathway [274].  $1,25(\text{OH})_2\text{D}_3$  and CYP11A1 derivatives of vitamin  $\text{D}_3$  and lumisterol have been reported to decrease NF- $\kappa$ B activation and inflammatory cytokines in keratinocytes after UV exposure [79,154,199]. Not surprisingly, given these results, it has been consistently reported that topical treatment with  $1,25(\text{OH})_2\text{D}_3$ ,  $20(\text{OH})\text{D}_3$ ,  $1,25$ -dihydroxylumisterol $_3$ , and a CYP11A1 derivative of lumisterol,  $24(\text{OH})\text{L}_3$ , reduces UV-induced skin edema in mice [22,26,152]. There is evidence of an inverse linear relationship between serum  $25(\text{OH})\text{D}$  and inflammatory markers

such as C-reactive protein, at least below  $50 \text{ nmol/L}$  [275]. This suppression of inflammation by good vitamin D status has been postulated to partly explain the better prognosis for patients with melanoma who have higher  $25(\text{OH})\text{D}$  at baseline [120]. This reduction in local and systemic inflammation may contribute to reduced progression of skin cancers and/or melanoma [120].

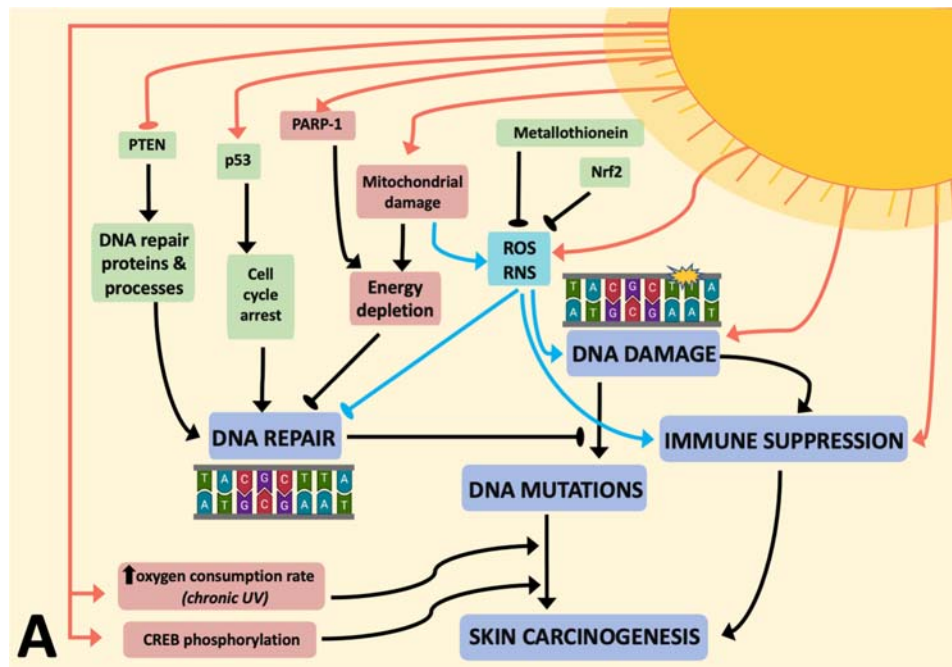
#### 4.7 Other potential contributors to reduced photo-carcinogenesis

Changes in energy metabolism are critical for neoplastic change and survival [276]. In particular, using the Skh:hr1 (hairless) mouse model of photo-carcinogenesis, these authors showed that the development of SCC-type skin tumors in these mice after chronic UV exposure was dependent on the upregulation of the distal part of the electron transport chain in the later stages of oxidative phosphorylation [276]. This was reflected in an increased oxygen consumption rate in keratinocytes derived from mouse skin after exposure to chronic UV [276]. This activation of the later stages of the electron transport chain was essential for synthesis of ATP and, importantly, pyrimidine nucleotides [276]. As noted earlier, single-dose UV acutely inhibited both glycolysis and oxidative phosphorylation [167]. While  $1,25(\text{OH})_2\text{D}_3$  increased glycolysis after a single dose of UVR, which was important for increased DNA repair at this time,  $1,25(\text{OH})_2\text{D}_3$  reduced oxygen consumption rate after UV and under basal conditions [166]. Given reports that other agents that reduce oxygen consumption rates such as leflunomide and metformin reduce pre-malignant and SCC-type tumors in mice after UV [276,277], it is not unreasonable to propose that this suppression of oxidative phosphorylation by  $1,25(\text{OH})_2\text{D}_3$  plays a role in reduction in skin tumors after chronic UV exposure.

A further UV-induced change in keratinocyte function is increased phosphorylation of cyclic AMP response-binding element protein (CREB) at Serine<sup>133</sup> at least in part as a result of UV stimulation of ERK $_{1/2}$  phosphorylation [166,278]. Inhibition of CREB phosphorylation by the plant-derived kaempferol in chronically UV-irradiated skin of Skh:hr1 mice markedly inhibited tumor formation [279]. Treatment of normal human keratinocytes with  $1,25(\text{OH})_2\text{D}_3$  inhibited ERK $_{1/2}$  phosphorylation and phosphorylation of CREB [166,167], which may also contribute to reduced photo-carcinogenesis.

## 5. Conclusions

An overall summary of the mechanisms likely to contribute to reductions in photo-carcinogenesis by  $1,25(\text{OH})_2\text{D}_3$  and related compounds is shown in



**FIGURE 93.6A** Adverse effects of UV radiation on skin cells. Exposure to UV causes damage by absorption of photons resulting in direct DNA damage as well as mitochondrial damage and generation of reactive oxygen and nitrogen species. UV activation of PARP-1, which inhibits glycolysis, as well as mitochondrial damage, leads to energy depletion. The ROS and RNS generated cause damage to DNA bases, contribute to immune suppression, and together with energy depletion inhibit DNA repair. DNA damage contributes to immune suppression. UV inhibits PTEN, which reduces DNA repair protein expression, but increases p53 expression in the nucleus, which leads to cell cycle arrest and facilitates DNA repair. Inadequately repaired DNA leads to mutations in key genes, which together with immune suppression results in skin neoplasia. UV exposure increases phosphorylation of CREB, while chronic UV results in increased activity of later stages of the electron transport chain providing energy and pyrimidine nucleotides important for neoplastic change.

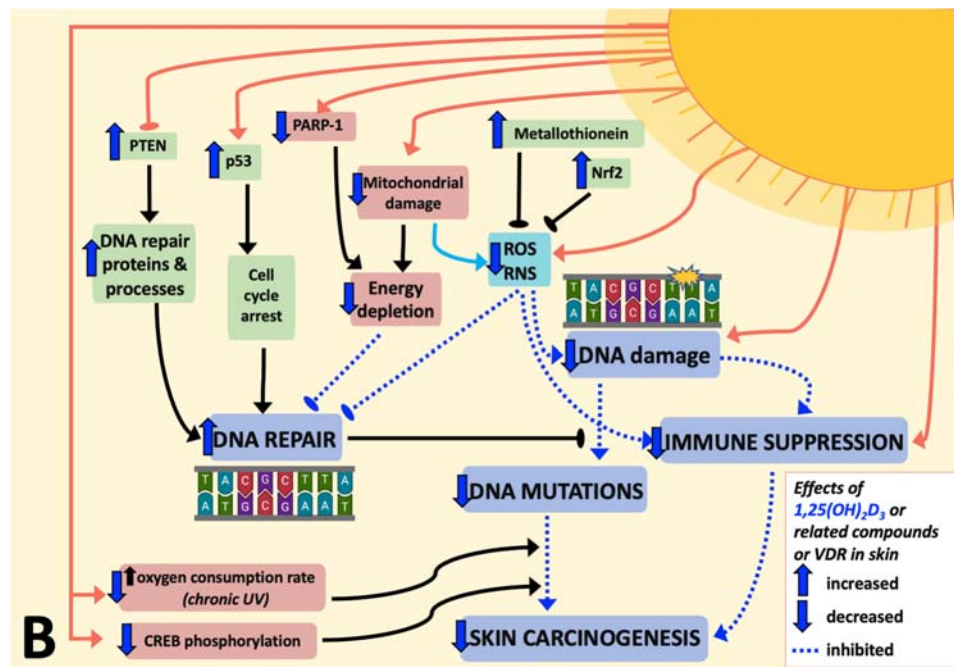
Fig. 93.6A,B. The hormone  $1,25(\text{OH})_2\text{D}_3$ , some naturally occurring related compounds, and specially synthesized derivatives predominantly alter cellular function by binding to the VDR and altering transcription [168,171]. The VDR may have functional effects without ligand. A variety of vitamin D compounds and many others, some of which are not cholesterol derivatives, including curcuminoids and other compounds, seem to be able to activate nonclassical pathways, possibly by binding to the alternate pocket on the VDR [150,155,171]. If, as the evidence suggests, nonclassical pathways contribute to photoprotection, many photoproducts of 7-dehydrocholesterol and their derivatives, including metabolites of vitamin D, such as  $25(\text{OH})\text{D}_3$  and the hormone, as well as CYP11A1 metabolites such as  $20(\text{OH})\text{D}_3$  and derivatives of overirradiation products such as  $24\text{-hydroxylumisterol}_3$  and related compounds, could contribute to reductions in UV-induced DNA damage and immune suppression. UV-adaptive mechanisms including increased pigmentation, increased thickness of the outer stratum corneum together with skin synthesis of vitamin D and related compounds are triggered by and develop after the first exposure to UV and so help to protect against subsequent exposures. Given this, it is highly likely that these

protective mechanisms would be more effective in practice if they developed gradually, under conditions of slowly increasing UV exposure during spring, as would normally be the case in hunter-gatherer societies.

## 6. Summary points

- Exposure of skin cells to UV causes DNA damage and immune suppression.
- Exposure of skin to UVB converts 7-dehydrocholesterol to pre-vitamin D, which converts to vitamin  $\text{D}_3$  at body temperature.
- 25- and  $1\alpha$ -hydroxylases in skin produce  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}_3$ .
- Both pre-vitamin D and vitamin  $\text{D}_3$  can absorb further UVB photons to form over-irradiation products, which are, in turn, acted on by CYP11A1 to form additional metabolites in skin.
- Vitamin D compounds in skin reduce UV-induced DNA damage by several mechanisms including reduced ROS/RNS, increased energy from glycolysis for repair, increased DNA repair proteins and processes, in part through increased PTEN and p53 and with a requirement for VDR and ERp57.





**FIGURE 93.6B** Effects of  $1,25(\text{OH})_2\text{D}_3$ , related compounds, and the VDR on skin cells following UV radiation. Working via the VDR and at least in part, requiring ERp57,  $1,25(\text{OH})_2\text{D}_3$ , and some related compounds reduce DNA damage by reducing reactive oxygen and nitrogen species at least in part through increased metallothionein, increased Nrf2 transcriptional activity, and more efficient mitochondrial repair. The vitamin D compounds also enhance DNA repair by both NER and BER, through increased provision of energy via glycolysis via reductions in PARP-1 activity, decreased ROS and RNS, maintenance of increased p53, and increased expression of repair proteins, partly through increased PTEN. These processes should result in decreased DNA mutations. The reduced DNA damage, increased DNA repair, and reduced ROS/RNS reduce UV-induced suppression of adaptive immunity. Fewer mutations and reduced immune suppression, together with reduced CREB phosphorylation and reduced activation of oxidative phosphorylation, result in less skin tumor formation after chronic UV exposure.

- Vitamin D compounds added topically reduce skin inflammation after UV in mice and reduce UV-induced immune suppression of the adaptive system in some mouse models in a sex-dependent manner.
- Mice that do not express VDR in skin are more susceptible to chemical- and UV-induced skin cancers.
- Applied topically after each dose of chronic UV,  $1,25(\text{OH})_2\text{D}_3$  and some vitamin D-like compounds, but by no means all, reduce UV-induced skin tumors in mice.
- VDR receptor polymorphisms and vitamin D status modify skin tumor development and prognosis in people in a complex manner.

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## Further reading

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# Vitamin D and antibacterial immunity

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## OBJECTIVES

- Present mechanisms of vitamin D–regulated antibacterial immunity as evidenced by in vitro data.
- Provide an update on our ongoing understanding of prominent antibacterial immune actions of vitamin D.
- Describe new, lesser-known antibacterial effects of vitamin D.
- Establish a mechanistic basis for the link between 1,25(OH)<sub>2</sub>D and antibacterial immunity with in vivo studies that have reported improved immune health with vitamin D supplementation.

## 1. Introduction

The hormonal form of vitamin D (calcitriol or 1,25(OH)<sub>2</sub>D) is best known for its role in calcium and phosphate homeostasis, as detailed in other chapters in this book. However, since the 1980s, the number of studies on the extraskeletal, so-called nonclassical actions of vitamin D has grown exponentially. In particular, these provide increasing evidence for a role of 1,25(OH)<sub>2</sub>D signaling in regulation of the immune system. In vertebrates, the immune system can be separated into the innate and adaptive arms. The function of vitamin D in overseeing adaptive immunity is covered in Chapter 96 by Hawrylowicz et al. Contrary to the adaptive immune system, innate immune responses are hard-wired and provide front-line protection upon

detection of pathogenic threat. They are initiated by signaling via so-called pattern recognition receptors (PRRs). PRRs detect various receptor-specific pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) [1]. PAMPs include components of bacterial cell walls or membranes and bacterial flagellin, while DAMPs, such as extracellular ATP, are endogenous molecules released in response to sterile inflammation. Among other responses, PRR signaling promotes the production of antimicrobial peptides, such as cathelicidin antimicrobial peptide (CAMP/LL-37) and  $\beta$ -defensin 2 (DEFB2/DEFB4/HBD2), which have direct antibacterial as well as antiviral activities. In humans, CAMP is produced initially as an 18-kDa protein, hCAP18, which is then cleaved to release the active 37-amino-acid peptide, LL-37 [2]. In addition, activation of PRRs induces the production of cytokines, which act to recruit other constituents of the immune system to sites of infection. As described in the following, expression of the genes encoding CAMP/LL-37 and HBD2 is directly induced by the hormone-bound VDR. Since this discovery, several studies have focused on the capacity of vitamin D to promote innate antimicrobial responses, which will be the main focus in this chapter. Antiviral activity of vitamin D will be covered in Chapter 95.

Interest in the nonclassical mechanisms of vitamin D action was stimulated by the observations that the vitamin D receptor (VDR) and the 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase enzyme (1 $\alpha$ -OHase) were expressed in various tissues not connected with calcium and phosphate homeostasis [3–5]. Notably, both the VDR and 1 $\alpha$ -OHase are expressed in cells of the innate and adaptive arms of the immune system [6–8] (see Chapter 9); in addition to its induction by PRR signaling, expression of

*CYP27B1*, the gene encoding  $1\alpha$ -OHase, in immune cells is regulated by noncalcium inputs such as IFN- $\gamma$  and other cytokines including TNF- $\alpha$ , interleukin (IL)-1, and IL-2 [9]. The pathogen-dependent local production of  $1,25(\text{OH})_2\text{D}$  in immune cells was first demonstrated in 2000 by Overbergh et al., who found that, in response to bacterial lipopolysaccharide (LPS) and IFN- $\gamma$ , *Cyp27b1* expression in mice was induced in macrophages [7]. The bacterial ligand, LPS signals through Toll-like receptor 4 (TLR4), a member of a family of PRRs related to the protein encoded by the *Toll* gene in *Drosophila*. LPS signaling in human macrophages also upregulates expression of *CYP27B1* [8]. Consistent with these findings, increased *CYP27B1* expression and endogenous  $1,25(\text{OH})_2\text{D}$  production were also observed in human macrophages challenged with the ligand of TLR1/2 heterodimers to model immune responses to detection of *Mycobacterium tuberculosis* (*M.tb.*) [10].

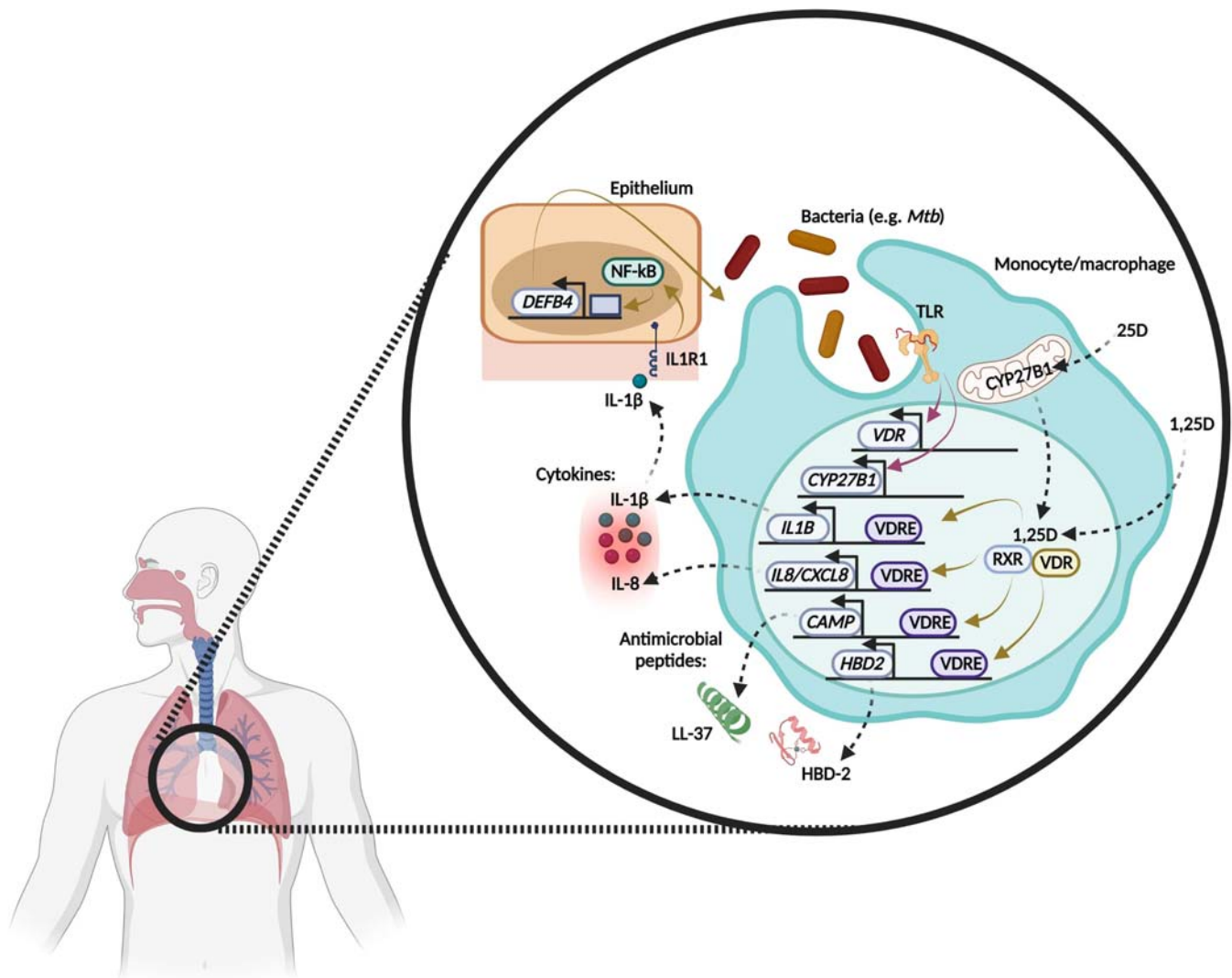
Associations between vitamin D status (or sunlight exposure) and infections have been established since the time of ancient Greeks, where heliotherapy (sun therapy) was often practiced to treat phthisis (tuberculosis, TB) [11]. Moreover, cod liver oil, a rich source of vitamin D, was first reported as a treatment for chronic rheumatism in the 18th century. Over the next century, vitamin D was also shown to be effective in treating gout and scrofula, a form of TB infecting the lymph nodes [12]. The notion of treating TB via sunlight became popular with the advent of the sanatorium movement in Europe in the mid-19th century, when incidence of the disease peaked [13,14]. Furthermore, Niels Finsen, who received the Nobel Prize for Medicine in 1903, demonstrated that exposure to UV light was efficacious against cutaneous TB, or lupus vulgaris [13,14]. A more in-depth focus on vitamin D signaling in tuberculosis is provided in Chapter 98 by Martineau et al. In more recent years, support for potential preventative roles for vitamin D in septic shock, autoimmune disease (such as type 1 diabetes and multiple sclerosis), and infectious diseases (for instance, respiratory tract infections), as well as cancer, has been provided by epidemiological observations [15–18]. Evidence from laboratory and preclinical studies examining the immune mechanisms of action of  $1,25(\text{OH})_2\text{D}$ , as well as clinical data on vitamin D supplementation and risk of immune disease, will be explored in the following section.

## 2. $1,25(\text{OH})_2\text{D}$ , barrier integrity, and complement activation

Maintenance of barrier integrity is critical to several immune processes [19]. The gut is comprised of a plethora of commensal bacteria and other immune

system-activating substances in the lumen that can lead to mucosal inflammation [20]. The body is kept clear of luminal microbes and inflammatory substances by the gut mucosal epithelial barrier. An impaired gut mucosal barrier leads to an increased risk of inflammatory bowel disease (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC) [21].  $1,25(\text{OH})_2\text{D}$  signaling has been shown to be critical in barrier homeostasis (see Chapter 97). This was most clearly demonstrated by the research group of Yan Chun Li and others, who showed that widespread deletion of the *Vdr* compromised mucosal barrier integrity, which led to increased mortality in a dextran sulfate sodium (DSS)–induced colitis model [22–24]. In the DSS model, *Vdr* knockout mice presented decreased colonic transepithelial electrical resistance, an established marker of epithelial barrier integrity [23]. Moreover, human *VDR* transgenic mice, which exhibit a 2–3 fold increase in *VDR* expression in the gut, displayed substantial resistance to colitis relative to wild-type control mice [25]. To show that epithelial-specific *VDR* has a role in anticolic activity, Li et al. reconstituted gut epithelial cells from human *VDR* transgenic mice in *Vdr* knockout mice. Following introduction of the *VDR* to *Vdr*<sup>−/−</sup> gut epithelial cells, the resulting mice were highly resistant to colitis, suggesting epithelial *VDR* signaling is accountable for preventing mucosal inflammation [25]. The cause of increased mucosal permeability in the *Vdr*<sup>−/−</sup> mice was attributed to increased gut epithelial cell apoptosis [26]. Interestingly,  $1,25(\text{OH})_2\text{D}$  suppresses intestinal epithelial cell apoptosis via inhibition of NF- $\kappa\text{B}$  activity, which is thought to be the main mechanism by which the hormone maintains intestinal barrier integrity [25].

In addition, the complement system is an integral component of innate immunity critical to enhancing (complementing) the capacities of antibodies and immune cells to clear pathogens. It also modulates adaptive immune responses. Briefly, in response to pathogen- or danger-sensing immune complexes (classical pathway) or danger-sensing (lectin pathway), the proenzyme complement 3 (C3) is activated to produce active C3a and C3b proteolytic fragments [27]. C3a and b in turn recruit and trigger activation of immune cells and downstream components of complement (C5–C9) [27]. Notably,  $1,25(\text{OH})_2\text{D}$  exerts an effect on this facet of innate immunity. In macrophages, mRNA and protein expression of complement receptor immunoglobulin (CRIg), responsible for phagocytosis and bacterial clearance, is upregulated in response to  $1,25(\text{OH})_2\text{D}$  [28]. This increase was linked with an amplification in phagocytosis of complement opsonized bacteria and fungi. In addition,  $1,25(\text{OH})_2\text{D}$  appears to attenuate the effects of complement signaling on chemotaxis of neutrophils, the first innate immune cells to sites of inflammation and infection. Function of the complement factor 5a (C5a),



**FIGURE 94.1** Antibacterial activity of vitamin D in response to infections in the monocyte/macrophage. See text for details.

chemotactic for neutrophils, is enhanced by the transport protein for vitamin D (vitamin D binding protein or DBP) [29]. However, binding of DBP to physiological concentrations of 1,25(OH)<sub>2</sub>D counteracted this neutrophil chemotaxis [30]. Consistent with its antiinflammatory role, 1,25(OH)<sub>2</sub>D can also modulate inflammatory responses of adaptive CD4<sup>+</sup> type 1 helper (T<sub>H</sub>1) cells following complement activation [31]. Complement was shown to mediate retraction of T<sub>H</sub>1 cell responses via induction of VDR and CYP27B1 expression. The ensuing 1,25(OH)<sub>2</sub>D signaling promoted the conversion of proinflammatory interferon-γ<sup>+</sup> T<sub>H</sub>1 cells to suppressive interleukin-10<sup>+</sup> cells. Intriguingly, bronchoalveolar lavage fluid CD4<sup>+</sup> T cells of patients afflicted with COVID-19 demonstrated derepression of genes downregulated by vitamin D [31].

### 3. 1,25(OH)<sub>2</sub>D induces antimicrobial peptides implicated in antibacterial immunity

1,25(OH)<sub>2</sub>D can directly contribute to the host responses against pathogens by activating the

transcription of a number of genes encoding proteins implicated in innate immunity, including antibacterial peptides (AMPs), pattern recognition receptors, cytokines, and components of autophagic responses (Fig. 94.1). In 2004, our laboratory found vitamin D response elements (VDREs) near the transcription start sites of two genes that encode the AMPs cathelicidin antimicrobial peptide (CAMP/LL-37) and β-defensin 2 (DEFB2/DEFB4/HBD2) [32]. Following 1,25(OH)<sub>2</sub>D treatment, CAMP expression was robustly induced in myeloid and bronchial epithelial cells, as well as keratinocytes [10,33,34]. CAMP transcription was also enhanced by 1,25(OH)<sub>2</sub>D in monocytes exposed to *M.tb*. [35]. Notably, activation of cathelicidin is suggested as a likely pathway through which 1,25(OH)<sub>2</sub>D induces innate immune responses to *M.tb*., as reported by studies using short interfering RNA to block 1,25(OH)<sub>2</sub>D-mediated CAMP expression [36].

Although only modest direct induction of DEFB4 was observed in cells treated with 1,25(OH)<sub>2</sub>D alone, 1,25(OH)<sub>2</sub>D substantially enhanced DEFB4 transcription induced by interleukin (IL)-1β [32]. Mechanistically,



signaling by IL-1 $\beta$  promotes binding of NF- $\kappa$ B transcription factor members to promoter-proximal sites in the *HBD2* promoter [37]. In accordance with these findings, exposure to 1,25(OH) $_2$ D promoted secretion into conditioned media antibacterial activity against *Escherichia coli* and the lung pathogen, *Pseudomonas aeruginosa* [32]. The induction of AMPs by 1,25(OH) $_2$ D may confer protection against other bacterial and nonbacterial pathogens, as human cathelicidin was reported to inhibit HIV-1, Ebola, and influenza A viral replication [38–40], and expression of defensins is augmented upon infection by *Helicobacter pylori* and rhinovirus [41,42]. Further, beta defensins were shown to inhibit HIV-1 replication in macrophages [43] and biofilm production of *P. aeruginosa* [44].

The link between 1,25(OH) $_2$ D-mediated *CAMP* transcription and protection against bacterial infections has been reinforced by several publications. For instance, topical administration of 1,25(OH) $_2$ D to the skin of transgenic mice carrying the human *CAMP* gene under control of its own promoter enhanced *CAMP* expression and augmented killing of *Staphylococcus aureus* as part of a model of wound infection [45]. Moreover, in respiratory epithelial (A549) cells infected with human rhinovirus (RV-16), pretreatment with physiological concentrations of 25(OH)D reduced expression of the platelet-activating factor receptor, involved in adhering *Streptococcus pneumoniae* to respiratory cells [46]. Notably, this decrease was correlated with a robust induction of *CAMP* expression, providing a mechanism by which vitamin D can attenuate the risk of secondary bacterial infection in vitamin D–deficient individuals infected with rhinovirus [46]. An additional role for 1,25(OH) $_2$ D-induced *CAMP* in enhancing pregnant innate immune defenses was suggested by Chin-Smith et al. [47]. In their study, 1,25(OH) $_2$ D was found to promote *CAMP* expression in the presence of inflammatory agents IL-1 $\beta$  and LPS in human endocervical epithelial cells [47].

Hepcidin antibacterial protein (HAMP) is another AMP regulated by 1,25(OH) $_2$ D. Although there is no direct microbial activity of HAMP, it was shown to inhibit ferroportin-facilitated export of iron and contribute in anemia of infection or inflammation. Diminishing level of iron in the circulation is a critical immune response to systemic infection, as several microorganisms require iron for their growth [48]. Further, infectious and immune stimuli enhance expression of HAMP, as demonstrated by mouse models of *Candida albicans* and influenza A/PR/8/34 virus infections [49]. Interestingly, Hewison and coworkers found that the 1,25(OH) $_2$ D-bound VDR transcriptionally inhibited *HAMP* expression in monocytes and hepatocytes [50]. Similar results were also obtained by Zughaier et al.,

who found increasing concentrations of 1,25(OH) $_2$ D correlated with suppressed hepcidin mRNA expression [51]. A decrease in *HAMP* induction was accompanied by an increase in the expression of ferroportin and a decrease in ferritin expression, which provides an indication for levels of intracellular iron [50]. Contrary to DEFB4 and *CAMP*, increased circulating 25(OH)D levels are associated with decreased amounts of serum HAMP. Nevertheless, like with defensins and cathelicidin, 1,25(OH) $_2$ D binds to specific VDREs in the *HAMP* promoter to directly repress its expression [50].

#### 4. 1,25(OH) $_2$ D and autophagy

Vitamin D signaling may also combat intracellular infections via induction of autophagy (Fig. 94.1). A compelling example of a role for vitamin D–induced autophagy is in infections with *M.tb.*, the etiological agent of tuberculosis. In macrophages, phagosome-resident *M.tb.* can escape host antimicrobial mechanisms via inhibiting the maturation of phagosomes and their fusion with lysosomes [52]. To conquer this evasion strategy, the host must degrade phagosomes containing bacteria via autophagy and subsequently destroy the bacteria in autolysosomes [53,54]. Interestingly, 1,25(OH) $_2$ D-mediated *CAMP* production enhanced autophagy in macrophages infected with mycobacteria, as demonstrated in an in vitro study by Yuk et al. [55]. The authors subsequently found that blocking 1,25(OH) $_2$ D-mediated autophagy with pharmacological inhibitors or siRNA against *CAMP* led to an increase in bacterial burden in infected macrophages. This study showed for the first time that physiological amounts of 1,25(OH) $_2$ D can initiate autophagy in infected macrophages to promote the elimination of *M.tb.* Further support for 1,25(OH) $_2$ D-induced *CAMP* in promotion of autophagy came from studies that revealed, in 1,25(OH) $_2$ D-exposed cells, *CAMP* was colocalized with mycobacteria in phagolysosomes [56]. In another study, autophagy was induced by 1,25(OH) $_2$ D in TLR2/1-agonist-stimulated macrophages isolated from 25(OH)D-deficient human populations, although the role of *CAMP* was not assessed [57].

Apart from induction of *CAMP* expression, there exist other mechanisms by which 1,25(OH) $_2$ D can trigger autophagy. 1,25(OH) $_2$ D-bound VDR also induces expression of the PRR NOD2, as well as ATG16L1, whose functions are linked to autophagy [58,59] (Fig. 94.1). Interestingly, the two proteins are connected, as NOD2 bound to muramyl dipeptide, the lysosomal breakdown product of bacterial peptidoglycan, recruits ATG16L1 to the plasma membrane upon bacterial entry and prompts subsequent autophagy and pathogen

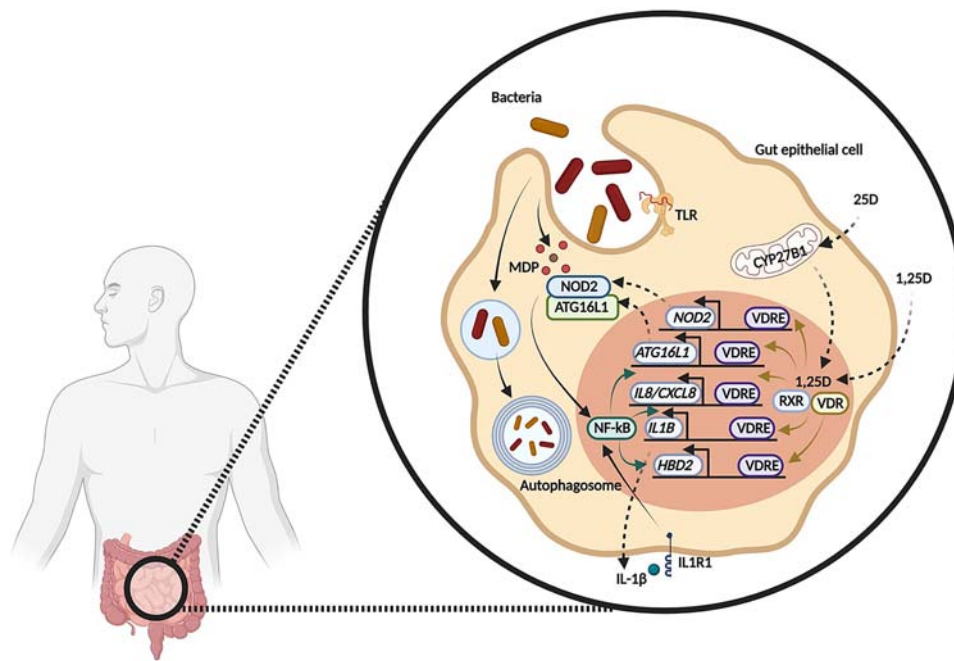
clearance [60] (Fig. 94.1). However, this process was abrogated in cells homozygous for mutations in *NOD2* or *ATG16L1* [60]. These results are highly significant as *NOD2* and *ATG16L1* have been identified as susceptibility loci in genome-wide association studies of patients with Crohn's disease, relapsing-recurring IBD [61–65]. This links (loss of) vitamin D signaling to CD, and CD to impaired autophagic responses to intestinal bacterial flora. Other studies also link VDR function to autophagy. Murine intestinal epithelium lacking the *Vdr* presents an imbalance of autophagy and apoptosis [66,67]. Decreases in lysozymes and autophagic responses, as defined by reduced expression of *ATG16L1* and the autophagosome-marker LC3, were reported in IBD-susceptible, Paneth cell-specific VDR knockout mice [68]. The impaired autophagy presented by these mice was associated with depleted intestinal microbiota and prolonged proinflammatory cytokine production, as well as decreased bacterial clearance in the context of *Salmonella* infection [68]. In addition, the *Vdr* is a master transcriptional regulator of autophagy in normal and tumorigenic mammary gland cells [69]. Furthermore, our laboratory showed that 1,25(OH)<sub>2</sub>D suppressed activation of a repressor of autophagy, the major metabolic kinase mammalian target of rapamycin (mTOR) in macrophages. This regulation was dependent on the activation of branched-chain amino acid (BCAA) catabolism [70]. BCAAs are essential amino acids and act as metabolic signals in myeloid cells. High BCAA levels activate mTOR, which enhances metabolically expensive processes such as protein synthesis and suppresses autophagy. 1,25(OH)<sub>2</sub>D-induced autophagy was found to be abolished in cells in which the gene encoding branched-chain aminotransferase, *BCAT*, the rate-limiting catabolizing enzyme of BCAAs, was ablated [70]. This finding is corroborated by a previous report on synthetic 1,25(OH)<sub>2</sub>D analogs inhibiting mTOR activity, resulting in autophagy in various cancer cell lines [71]. Further, a study by Song et al. found that 1,25(OH)<sub>2</sub>D treatment restored autophagy, via induction of autophagy-associated proteins such as Beclin-1, in podocytes from patients with diabetic nephropathy compared with healthy controls [72].

## 5. 1,25(OH)<sub>2</sub>D promotes expression of pattern recognition receptors and cytokines

Another facet of 1,25(OH)<sub>2</sub>D signaling in innate immunity is encompassed by the regulation of PRR and cytokine expression (Fig. 94.1). In the early 1990s, the gene that encodes the TLR4 coreceptor, *CD14*, was determined to be a target gene of 1,25(OH)<sub>2</sub>D [73]. *CD14*

serves as a coreceptor for multiple other TLRs, such as TLR1/2 [74], which, like TLR4, responds to bacterial ligands, as well as the viral-sensing TLRs, TLR7 and TLR9 [75]. Following TLR activation, antimicrobial peptides and reactive oxygen species (ROS) are induced, which help to destroy microorganisms. Although excessive ROS can result in cellular damage, 1,25(OH)<sub>2</sub>D can keep this in check by promoting expression of antioxidant genes such as glutathione synthase [76]. Moreover, 1,25(OH)<sub>2</sub>D upregulates the intracellular pathogen-sensing protein *NOD2/CARD15/IBD1* in myeloid and epithelial cells [77]. Thus, in cells pretreated with 1,25(OH)<sub>2</sub>D to induce *NOD2*, its cognate ligand muramyl dipeptide superinduces *CAMP* and *DEFB4* expression via induction of NF-κB signaling. However, this cooperative induction of *DEFB4* is eliminated in patients carrying inactivating *NOD2* mutations afflicted with CD [77]. Individuals who are heterozygous or homozygous for nonfunctional mutations of the *NOD2* gene bear higher risks of developing CD by 3-fold and 20-fold, respectively [78]. Moreover, the *DEFB4* locus has undergone a series of gene duplication events, and individuals with low copy number of *DEFB4* are also at increased risk of developing colonic CD [79]. This implicates defective induction of the *NOD2-DEFB4* pathways as a risk factor for CD.

1,25(OH)<sub>2</sub>D may also repress expression of PRRs, as shown in a study of *M.tb.*-infected human peripheral blood mononuclear cells, where 1,25(OH)<sub>2</sub>D-bound VDR-repressed TLR2, TLR4, dectin-1, and mannose receptor [80]. This suppresses the production of proinflammatory cytokines IL-6, TNF-α, and IFN-γ, consistent with an antiinflammatory role for 1,25(OH)<sub>2</sub>D signaling. IL-6 and TNFα were also inhibited by 1,25(OH)<sub>2</sub>D in LPS-treated human and murine monocytes and macrophages [81,82]. Further, 1,25(OH)<sub>2</sub>D synergistically functions with other signals to modulate cytokine expression; for instance, glucocorticoid-induced suppression of IL-6 in LPS-challenged human monocytes was enhanced upon 1,25(OH)<sub>2</sub>D treatment [83]. However, not all cytokines and chemokines regulated by 1,25(OH)<sub>2</sub>D are repressed, which serves to modulate the immune response. IL-1β, a cytokine secreted in response to infection, is directly induced by the hormone in human myeloid cells [84]. The functional importance of this was shown in a coculture of primary human airway epithelial cells with macrophages infected with *M.tb.*; this coculture system revealed that 1,25(OH)<sub>2</sub>D- and *M.tb.*-induced IL-1β secretion prolonged survival of infected macrophages and decreased *M.tb.* burden in those cells by inducing *DEFB4* production in the cocultured lung epithelial cells. 1,25(OH)<sub>2</sub>D also robustly upregulated the chemokines, *CCL3*, *CCL4*, and *CCL8*,



**FIGURE 94.2** Mechanisms of vitamin D signaling following exposure to bacteria in the gut epithelial cell. See text for details.

as well as the neutrophil chemoattractant, IL-8/CXCL8 [84]. In addition,  $1,25(\text{OH})_2\text{D}$  can indirectly promote cytokine expression through CAMP induction; LL-37, the active peptide encoded by CAMP, can trigger the release of antiinflammatory IL-10 and proinflammatory IL-18 cytokines [85]. A summary of vitamin D signaling in the monocyte and gut epithelial cell is provided by Figs. 94.1 and 94.2, respectively.

## 6. $1,25(\text{OH})_2\text{D}$ regulates release of reactive oxygen species

$1,25(\text{OH})_2\text{D}$  can also enhance bacterial killing through induction of ROS, which are defined by a class of endogenous, highly reactive, oxygen (and nitrogen)-bearing molecules that are directly antimicrobial. In the presence of  $1,25(\text{OH})_2\text{D}$ , monocytes infected with *M.tb.* generate an increased amount of bactericidal superoxide anions [86]. Further,  $1,25(\text{OH})_2\text{D}$ -mediated repression of *M.tb.* growth in monocytes may be associated with production of nitric oxide (NO) [87,88], which has been previously shown to serve a critical bactericidal mechanism in infected mice [89]. However,  $25(\text{OH})\text{D}$  deficiency resulted in increased ROS release and DNA damage, which consequently led to exacerbated asthma [90], a condition where the innate immune system plays a critical role [91]. The same study reported vitamin  $\text{D}_3$  inhibited LPS-induced ROS, which was associated with decreased levels of  $\text{TNF-}\alpha$  and  $\text{NF}\kappa\text{B}$  in epithelial

cells [90]. The role of vitamin D in asthma is explored further in Chapter 105 by Dr. Camargo.

## 7. Evolutionary aspects of $1,25(\text{OH})_2\text{D}$ induction of AMPs and PRRs

It is important to note that many mechanisms of  $1,25(\text{OH})_2\text{D}$ -mediated innate immune signaling appear to be limited to humans and primates. This was made clear through observation of conserved VDREs in *HBD2*, *CAMP*, and *NOD2* genes in primates, but not in rodents [92]. Notably, the VDRE in the *CAMP* gene is present within an Alu repeat element existing only in primate and human genomes [33]. The transposition event occurred before the evolutionarily split Old and New World monkeys [93]. Further support for the notion of species specificity emerged from in vitro studies where no induction of *Defb2*, *Cramp* (the murine homolog of *CAMP*), and *Nod2* genes was observed in  $1,25(\text{OH})_2\text{D}$ -treated murine cells of epithelial or myeloid origin. In addition, unlike in control human cells, induction of antimicrobial activity against *E. coli* or *P. aeruginosa* was undetectable in  $1,25(\text{OH})_2\text{D}$ -treated mouse epithelial cells [33]. However, it is noteworthy that in mice lacking *Cyp27b1*, *Cd14* induction by  $25(\text{OH})\text{D}$  was abrogated [94], and agonists of TLR signaling promoted murine macrophage *Cyp27b1* expression [7]. Thus, the TLR coreceptor gene *Cd14* appears to be a VDR target gene in human and mouse.



Separate mechanisms of 1,25(OH)<sub>2</sub>D regulation of AMPs in humans and mice can be attributed to the nocturnal nature of mice, while humans are diurnal and, as a result, can synthesize active vitamin D from sunlight [10]. The expanded role of 1,25(OH)<sub>2</sub>D in innate immunity of humans/primates may have evolved as a protective response to sun exposure. Further, differences in human and murine immune systems can be attributed to distinct sites of 1,25(OH)<sub>2</sub>D synthesis, as CD8<sup>+</sup> T cells rather than macrophages are suggested as the primary immune cell source of *Cyp27b1* expression and activity in mice [95].

## 8. 1,25(OH)<sub>2</sub>D and neutrophils

The majority of studies thus far have explored regulation of the innate immune response by vitamin D in cells of monocytic or epithelial origin. Yet, there are a number of other immune cell types that express PRRs and hence carry the essential machinery required to initiate innate immune reactions to microbial threat. Neutrophils are the most numerous among the granulocyte population, and patients with congenital neutrophil insufficiencies are frequently afflicted with severe infections, emphasizing the significance of this cell type in immune defense [96]. Upon microbe invasion, these short-lived cells become “activated,” a term referring to the neutrophil’s integration of environmental signals and translation into specific actions. During its pursuit of pathogens, the neutrophil mobilizes secretory vesicles and granules, traverses via chemotactic gradients to sites of infection/inflammation, and starts transcribing cytokine genes to recruit more immune cells and releasing its arsenal of antimicrobial defense. Neutrophils also contain and produce a large amount of cytotoxic molecules; this is mainly on account of factors stored in their granules, such as antibacterial enzymes (e.g., myeloperoxidase [97]) and peptides (e.g., defensins [98]), in addition to serine proteases (e.g., neutrophil elastase [99]). Methods of microbial killing by neutrophils include phagocytosis, degranulation, production of ROS (respiratory burst), and release of neutrophil extracellular traps (NETs). NETs are structures of extracellular fibers composed of DNA, citrullinated histones (H3Cit), and antimicrobial enzymes that function to immobilize pathogens [96]. However, the killing of invading microorganisms by these cells requires careful control of neutrophil function, as neutropenia results in vulnerability to infection, whereas overactivity is linked with inflammatory diseases [100]. Phagocytosis generally expedites neutrophil apoptosis, which eventually results in the resolution of infection or inflammation [101]. Conversely, a

number of bacteria modify and inhibit neutrophil apoptosis to extend their survival and hence cause disease [101].

The literature to date on the effect of vitamin D on neutrophil biology is not extensive. Human neutrophils express *VDR* mRNA to a similar extent to monocytes, and upon exposure to 1,25(OH)<sub>2</sub>D, promote *CD14* and *CAMP* expression [32,102]. However, contrary to monocytes, it is not apparent that these granulocytic cells express *CYP27B1*, suggesting that they are more likely to respond systemically to circulating hormonal 1,25(OH)<sub>2</sub>D. Neutrophils may be exposed to paracrine 1,25(OH)<sub>2</sub>D produced via innate immune signaling described before at sites of infection. Regardless, because of their abundance and development of granules that carry the bulk of LL-37 secreted at sites of infection, neutrophils are the predominant source of serum cathelicidin [103,104]. In patients with chronic kidney disease, there is a strong link between decreased serum LL-37 and mortality, and levels of LL-37 correlated with serum 1,25(OH)<sub>2</sub>D, rather than 25(OH)D. This may represent an endocrine-mediated immune response involving neutrophils with low *CYP27B1* and high *VDR* expression [105,106]. However, this is not universal, as in septic patients with a sustained high numbers of neutrophils, circulating LL-37 is lower in severe cases and is linked with low serum 25(OH)D [107].

Takahashi et al. in the early 2000s found that 1,25(OH)<sub>2</sub>D modestly suppressed LPS-induced neutrophil elastase inhibitor trappin-2/elafin/SKALP and IL-1β in human primary neutrophils [102]. Further, increased apoptosis in COPD patient neutrophils was observed after 1,25(OH)<sub>2</sub>D treatment [108]; this is remarkable given that a decreased rate of neutrophil apoptosis is characteristic of the disease, and vitamin D offset granulocytic cell death through activation of p38 MAPK. The hormone was also reported to strongly boost IL-8 secretion in *M.tb.*-infected macrophages [84] as well as LPS-challenged neutrophils [109]; however, no impact on phagocytosis was noted when cells were infected with *E. coli* [109]. On the contrary, 1,25(OH)<sub>2</sub>D can inhibit neutrophil degranulation as determined by release of myeloperoxidase activity and ROS production in an inflamed mouse model of carrageenan-induced paw edema [110]. Moreover, neutrophil killing of the bacterium, *Streptococcus pneumoniae*, was increased with 1,25(OH)<sub>2</sub>D treatment, and expression of the antiinflammatory cytokine IL-4 and suppressor of cytokine signaling (SOCS) proteins were induced by the hormone, suggesting a role for vitamin D in inhibiting neutrophil-mediated inflammatory responses while still promoting microbial killing [111]. Agraz-Cibrian et al.’s pilot study showed that 1,25(OH)<sub>2</sub>D enhanced formation of NETs [112].



However, it should be noted that the structures observed were not confirmed to be genuine NETs in this study [112].

### 9. 1,25(OH)<sub>2</sub>D and natural killer cells

Natural killer (NK) cells are often reported to serve as the link between innate and adaptive immunity [113]. Although there are studies suggesting no effect of 1,25(OH)<sub>2</sub>D on NK cells [114–116], others indicate a role for the hormone in this cell type [117–125]. Dysregulated NK cell activity is a hallmark of patients with vitamin D metabolic disorders such as vitamin D-resistant rickets and chronic renal failure [117,118]. Upon supplementation with vitamin D, normalized activity of NK cells began to appear in these patients. Further, low serum 25(OH)D was linked with impaired NK cytolytic function among a population of healthy elderly people [121]. In vitro studies corroborate the in vivo results, as treatment with 1,25(OH)<sub>2</sub>D dose-dependently increased the number of cellular granules and reduced the delay in granzyme A secretion in NK cells, both markers of enhanced NK activity [119,122]. In addition, a regulatory NK cell phenotype, where T cell responses are repressed, was induced by 1,25(OH)<sub>2</sub>D; this was made evident with upregulated antiinflammatory IL-10 cytokine mRNA in response to the sunshine hormone and glucocorticoid dexamethasone in NK cells [120]. Along with regulating proinflammatory NK function, a contribution by 1,25(OH)<sub>2</sub>D to enhancing NK cell cytotoxicity was also suggested [123–125].

### 10. 1,25(OH)<sub>2</sub>D and nonclassical immune cells

Antibacterial activity of vitamin D has also been depicted in cell types extrinsic to the classical immune system. For instance, as previously mentioned, 1,25(OH)<sub>2</sub>D promotes increased expression of LL-37 in keratinocytes [33]. However, in contrast to monocytes, the mechanism behind this is not related to TLR2-TLR1 activation. Rather, soluble factors, including transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), are necessary to induce expression of *CYP27B1* and local production of 1,25(OH)<sub>2</sub>D [94]. The increased concentrations of 1,25(OH)<sub>2</sub>D and *VDR* expression subsequently promote induction of *TLR2* gene expression in keratinocytes. The ensuing high levels of *TLR2* allow increased responsiveness to infectious agents and additional *CYP27B1* and *VDR* expression mediated by a TLR2-dependent mechanism [94]. In this manner, there is vitamin D-induced intracrine production of cathelicidin in keratinocytes, similar to that observed in monocytes. Epithelial wound

healing represents a scenario in which sufficient TGF- $\beta$ 1 is produced to promote keratinocyte antibacterial activity. The necessity for a dual mechanism to enable vitamin D-dependent antibacterial responses in the skin may be attributed to the various functions of cathelicidin. Not only is cathelicidin antibacterial, but it is also shown to modulate antigen presentation [126] and epidermal wound healing [127]. However, cathelicidin induces release of cytokines and chemokines, which in turn can exacerbate tissue inflammation [128–130]. For a detailed overview of the role of vitamin D in the skin, readers are referred to Chapter 25.

In addition to keratinocytes, vitamin D-facilitated intracrine production of cathelicidin appears during pregnancy in placental decidual (maternal) [131] and trophoblastic (fetal) cells [132]. Interestingly, vitamin D-induced CAMP activity in these cells does not occur via the same TLR-mediated induction of *CYP27B1* reported in cells from the skin or immune system. This difference may be attributed to the expression of *CYP27B1* and *VDR* being induced early in gestation, contributing to a high baseline level of 1,25(OH)<sub>2</sub>D synthesized endogenously in the placenta [133]. During pregnancy, the placenta represents a natural barrier to fetal infection, and both maternal and fetal tissues express antimicrobial proteins [134]. Thus, robust 1,25(OH)<sub>2</sub>D production in the placenta may explain the high baseline expression of antibacterial proteins to protect against infection by pathogens, including *Listeria monocytogenes*, which is involved in adverse effects linked with pregnancy [135]. More information on the effect of vitamin D signaling in the placenta is provided by Chapter 34.

Further, the cornea of the eye is a site for local inflammatory responses required for efficient wound healing and is sensitive to vitamin D signaling. The function of the hormone in the eye is explored extensively in Chapter 42. Corneal cells contain *VDR* and *CYP27B1* mRNA and show enhanced barrier function upon exposure to 1,25(OH)<sub>2</sub>D in vitro [136]. Improved barrier activity with 1,25(OH)<sub>2</sub>D treatment is also observed in an in vivo mouse model of corneal epithelial wound healing, and topical administration of the hormone increased production of murine c 12 h following wounding [137]. Increased CAMP mRNA expression following 1,25(OH)<sub>2</sub>D treatment was also reported in human corneal epithelial cells and was accompanied by a decrease in proinflammatory cytokine expression [138].

### 11. In vivo evidence for 1,25(OH)<sub>2</sub>D regulation of antibacterial innate immunity

There is ample clinical data supporting a role for 1,25(OH)<sub>2</sub>D in the regulation of antibacterial innate immunity. For example, antimicrobial activity of lung

airway surface liquid (ASL) was enhanced by supplementation with 1000 international units (IU; 25 µg) per day for 90 days of vitamin D, as reported by a single-center community-based randomized placebo-controlled double-blind trial [139]. In addition, enhanced antimicrobial activity in ASL was reported in the summer–fall season compared with winter–spring, and an anti-LL-37 antibody blocked ASL antimicrobial activity. Moreover, supplementing with vitamin D eliminated the seasonality of ASL antimicrobial activity [139]. The clinical significance of 1,25(OH)<sub>2</sub>D-induced CAMP production was also illustrated by a Swedish study that found a link between inadequate vitamin D levels and an elevated risk of dental caries [140]. Antimicrobial peptide production is critical for prevention of dental caries; decreased levels of antimicrobial peptides [141] and presence of *DEFB1* promoter polymorphisms [142] are linked with increased risk of caries. In the Swedish study, concentrations of CAMP in saliva correlated positively with vitamin D status in participants [140]. Likewise, a prospective cohort study in Spain found the risk of caries is tripled when 25(OH)D values were lower than 20 ng/mL among 188 children between 6 and 10 years of age [143]. This finding was also corroborated by cross-sectional, observational and randomized clinical investigations that place vitamin D deficiency as a risk factor for dental caries [144–146]. Similarly, a large-scale umbrella analysis of systematic reviews and metaanalyses of observational studies and randomized trials concluded that poor vitamin D status is a risk factor for dental caries [145]. Importantly, in this regard, CAMP/LL-37 is efficacious against bacterial species that are found in plaque, such as *Streptococcus mutans* [147]. In addition to dental caries, serum vitamin D concentration is linked with antimicrobial peptide levels in other diseases of oral health. For instance, gingivitis and chronic periodontitis patients who were vitamin D–deficient (25(OH)D levels of <20 ng/mL) presented decreased expression of HBD2 and LL-37, as determined by ELISA [148].

Poor serum 25(OH)D status is also associated with increased risk of urinary tract infections (UTI). In the urinary bladder, vitamin D signaling promotes CAMP expression [149], and urinary LL-37 is correlated with vitamin D status in children less than 3 years of age [150]. Clinically, vitamin D deficiency is linked with recurrent rates of UTI in infants, children, and premenopausal women [150–153]. A systematic metaanalysis including nine studies and 1921 participants, of which 580 were diagnosed with UTI, revealed that vitamin D deficiency was more prevalent in the UTI group [154]. The same study verified the robust correlation between vitamin D insufficiency and UTI in children [154]. Moreover, UTI is causally linked with the most common liver disease linked with obesity in the West: nonalcoholic

fatty liver disease (NAFLD) [155], which is exacerbated by vitamin D deficiency [156,157] and improved with vitamin D treatment [158]. Interestingly, there is a strong link between levels of gut microbial metabolites, such as bacterial-derived ethanol, and NAFLD progression [159,160]. Consistent with this, vitamin D affects intestinal innate immunity and gut microbial structure, for example, by regulating mucin gene expression [161] and ameliorating mucosal impermeability to bacterial endotoxin infiltration [162], which may provide a mechanism for its role in NAFLD. In addition, there is indirect evidence for AMPs regulated by vitamin D in dampening NAFLD; in diabetic mice fed a high-fat diet, NAFLD and fat mass were reduced upon lentiviral-driven overexpression of cathelicidin [163].

In CD, the inflammatory bowel condition arising from impaired intestinal innate immunity, patients often present with low levels of vitamin D [164]. Notably, vitamin D metabolite levels were associated with a decreased risk of CD recurrence, as determined by endoscopy [165]. Two metaanalyses have reanalyzed several small clinical studies on CD and vitamin D supplementation. Li et al. found diminished rates of relapse among vitamin D–treated CD patients relative to controls [166]. Likewise, low 25(OH)D status was associated with amplified IBD activity, mucosal inflammation, and ensuing clinical relapse in CD patients in a systematic review by Gubatan and colleagues [167]. Interestingly, while mutations in the gene encoding the PRR NOD2, whose expression is inducible by 1,25(OH)<sub>2</sub>D [32], are associated with susceptibility to CD in Western patients, they are not detected in afflicted Korean, Japanese, or Chinese CD patients [168]. Nonetheless, a cohort investigation conducted in South Korea found aggravated disease course and increased risk of surgical intervention were associated with severe vitamin D deficiency (<10 ng/mL) [169], findings consistent with other clinical studies of vitamin D status in Asian CD patients [170,171]. Mental health disorders are also common among individuals affected with CD, and a systematic review of 10 intervention studies conducted by Glabska et al. determined a positive association between vitamin D supplementation and the mental health of CD patients [172].

Decreased levels of circulating 25(OH)D are also prevalent, and correlate with, disease severity in chronic obstructive pulmonary disease (COPD) [173–177], although, overall, the data are conflicting (Chapter 105). COPD is an airway inflammatory condition with chronic bacterial infection as one of its causes [178]. Support for the role of vitamin D in COPD was provided by a randomized, double-blinded, placebo-controlled trial, which recruited 442 adults to receive a monthly high-dose (100,000 IU or 2500 mcg) of vitamin D<sub>3</sub> for 1.1 years [179]. Although the study did not find significantly

improved lung function, as measured by decreased forced expiratory volume in 1 s (FEV1), in the total population, vitamin D<sub>3</sub> supplementation was of benefit in participants with existing lung problems such as asthma, COPD, or a history of smoking [179]. However, a metaanalysis of eight cohort and randomized controlled trials (RCTs) consisting of 687 COPD subjects determined no improvement in lung function with either short-term (<6 months) nor long-term (≥6 months) vitamin D<sub>3</sub> supplementation [180]. Another report combined three cycles of National Health and Nutrition Examination Surveys, and although, there was no relation between serum 25(OH)D and prevalence of asthma, chronic bronchitis, and emphysema among US adults, serum 25(OH)D was associated with ameliorated markers for lung effectiveness [181]. Overall, the clinical data for vitamin D supplementation in COPD is mixed, and additional large-scale, randomized and placebo-controlled controlled studies are required to assess the possible role of vitamin D in influencing the risk and course of COPD.

In addition, a low level of circulating vitamin D metabolites is likely to increase the risk for severity of sepsis, which is defined as the dysfunctional host response to an infection leading to organ failure. Vitamin D deficiency has been reported in several critically ill patients with sepsis, and interestingly, this was correlated with low amounts of LL-37 [182,183]. Patients with suspected infection and 25(OH)D insufficiency had higher sepsis severity, as measured by a sequential organ failure assessment greater than 2, than infected patients with sufficient vitamin D levels [184]. Vitamin D deficiency was a significant predictive marker of sepsis and displayed a 1.6-fold increase in mortality, as reported in a large two-center observational study including 3386 critically ill patients [185]. This is also corroborated by several studies on vitamin D and critically ill children with sepsis [186]. 25(OH)D deficiency is prevalent (64%) in children with sepsis and is linked with increased mortality, according to a systematic review of 18 studies evaluating 889 patients [187]. Moreover, low levels of vitamin D in cord and maternal blood were significantly associated with neonatal sepsis, based on a metaanalysis of 18 cohort and case-control studies [188]. Another metaanalysis found that vitamin D insufficiency was linked to a higher incidence of sepsis, severity of illness, and length of hospital stay [189]. However, other reports of pediatric sepsis and vitamin D deficiency demonstrate weak association with ventilation and mortality [190,191]. Furthermore, vitamin D levels in children with sepsis are not consistently correlated with levels of DBP or LL-37 [192–194], suggesting that mechanisms for enhanced outcomes with the hormone remain inconclusive.

Clinical trials regarding vitamin D supplementation and risk for sepsis severity and mortality are promising although the results are mixed. In a number of RCTs of critically ill adults, vitamin D supplementation was associated with ameliorated outcomes, reduced length of stay, and decreased mortality [195–197]. In contrast, a systematic review in adults found no link between length of stay, duration of mechanical ventilation, or mortality [198]. However, a single dose of 150,000 IU (3.75 mg) vitamin D in 109 children decreased inflammatory markers, dampened cardiovascular organ failure scores, and reduced progression to septic shock [199]. Additionally, a link between high-dose administration of 200,000 and 400,000 IU (5 and 10 mg) of vitamin D<sub>3</sub> and increased plasma 25(OH)D and LL-37 was observed in a group of 30 adult septic shock intensive care unit (ICU) patients [200]. Globally, a clear consensus of whether vitamin D supplementation can improve outcomes and is a modifiable risk factor for sepsis is challenging to discern due to various factors in study design, such as variabilities in sample sizes and heterogenous populations [198,201].

## 12. Conclusion

Since observations of vitamin D signaling in tissues unrelated to calcium and phosphate regulation in the early 2000s, a number of studies have focused on the role of the hormone in immune responses to date. The majority of in vitro studies on the antibacterial properties of vitamin D have focused on the capacity of the hormone to induce expression of antimicrobial peptides, such as CAMP, to promote autophagy as a mechanism for handling intracellular pathogens, and to enhance and regulate transcription of genes encoding pattern recognition receptors and cytokines. Generally speaking, the in vitro data is well supported by in vivo studies, as vitamin D supplementation has been demonstrated to clinically suppress risk of oral health diseases, urinary tract infections, relapse of Crohn's disease, and may be of benefit for COPD and sepsis, all of which are common immune conditions with dysregulated antibacterial activity.

Additional work uncovering the influence of 1,25(OH)<sub>2</sub>D in antibacterial activities of neutrophils, NK, NK T cells, and  $\gamma\delta$  T cells represent future avenues of investigation, as these innate immune cell populations are crucial in the killing of infected cells [113], immediate release of cytokines [202], and interaction with innate and adaptive cells to provide host defense [203]. Moreover, the significance of the sunshine hormone in regulating antibacterial immune function may motivate development of vitamin D analogs that may serve as constituents of combined therapies or even as front-line therapies for treating bacterial, antibiotic-resistant disease.



### 13. Summary points

- 1,25(OH)<sub>2</sub>D signaling affects susceptibility to and severity of bacterial infection via induction of antimicrobial peptides, pattern recognition receptors, and cytokines in innate immune cells.
- Intracellular bacterial infections, such as *M.tb.*, are suppressed via 1,25(OH)<sub>2</sub>D-stimulated autophagy.
- Preclinical and clinical reports strongly suggest an association between circulating 25(OH)D levels and vulnerability to conditions caused by bacterial infections, such as diseases of oral health, urinary tract infections, and Crohn's disease.
- Exploration of vitamin D supplementation as a therapeutic intervention may be clinically and economically important in the treatment of immune conditions of bacterial origin.

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## Further reading

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# Vitamin D and antiviral immunity

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## OBJECTIVES

- Discuss epidemiological association between vitamin D insufficiency and viral diseases.
- Consider potential of in vivo 1,25(OH)<sub>2</sub>D supplementation for improved antiviral immunity.
- Present mechanisms of 1,25(OH)<sub>2</sub>D-regulated antiviral immunity.
- Provide an update on recent advances in antiviral immune actions of 1,25(OH)<sub>2</sub>D in COVID-19.

## 1. Introduction

Viral infections pose a major public health challenge with over 40 million deaths worldwide ascribed to human immunodeficiency virus (HIV) infections since the beginning of the epidemic, over 6.5 million deaths due to the global severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) pandemic in the past 3 years, and significant disease burden due to other respiratory tract infections, such as influenza and rhinovirus, and chronic hepatitis and herpes viral infections based on the World Health Organization (WHO) tallies. Vitamin D, a key nutrient/prohormone classically associated with skeletal health, has emerged as a key immunomodulator, and insufficient levels of circulating

vitamin D (serum 25-hydroxyvitamin D, 25(OH)D) have been associated with increased occurrence of viral infections and disease severity.

Vitamin D may directly inhibit viruses through interaction with viral or cellular proteins necessary for infection, replication, or spread of the virus. For example, the biologically active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) suppresses HCV production in human hepatocytes by inhibiting the expression of apolipoproteins critical for virion assembly and by interfering with the viral nonstructural 3 (NS3) protein during viral assembly [1–6]. 1,25(OH)<sub>2</sub>D-induced autophagy or apoptosis is another mechanism of viral control in several infections such as HCV, HIV-1, rotavirus, and influenza A virus [7–12]. 1,25(OH)<sub>2</sub>D has also been found to inhibit HIV infection by downregulating chemokine receptor 5 (CCR5), which serves as a coreceptor for HIV-1 entry into the target cells [13], and subsequent viral replication in infected PBMC through the induction of phagosome maturation [7,14]. Likewise, 1,25(OH)<sub>2</sub>D-mediated repression of C-type lectin mannose receptor on macrophages has been shown to impair dengue virus infection [15]. 1,25(OH)<sub>2</sub>D mitigates virus-induced vascular endothelial damage during human cytomegalovirus (HCMV)-induced atherosclerosis [16]. Maintenance of barrier integrity at mucosal sites of viral infection is yet another nonimmune antiviral mechanism by vitamin D. 1,25(OH)<sub>2</sub>D signals through VDR to support mucosal barrier integrity [17–20], by possibly suppressing apoptosis through

a These authors contributed equally.

inhibition of NF- $\kappa$ B [21]. Contrarily, vitamin D has also been shown to induce HCMV growth in monocytic cells by altering a more favorable differentiation state [22]. Likewise, in the case of HIV-1 replication, vitamin D signals have been shown to promote viral LTR transactivation in vitro [23]. Viral proteins, in turn, have been shown to modulate the vitamin D status of host cells as a possible escape mechanism in the case of HBV [24] and EBV [25–27]. As opposed to these nonimmunological mechanisms of viral control by vitamin D, which are uniquely dependent on distinct virological parameters such as host cell types and propagative modes for each virus, the primary goal of this chapter is to present the innate and adaptive immune modulatory effects of vitamin D in the context of distinct viral infections.

The innate immune responses constitute the first line of antiviral defense with broad specificity [28–31]. Triggered by engagement and signaling of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) in virally infected target cells, the innate antiviral defense mechanisms directly target viral and cellular factors critical for viral propagation. Production of antiviral and proinflammatory cytokines (such as type I and type III interferons, TNF- $\alpha$ , IL-1 $\beta$ ) and chemokines (such as CCL-2/4, CXCL-8/9/10), serves to further activate and recruit additional innate immune cells through induction of numerous interferon-stimulated genes (ISGs), thus amplifying the innate antiviral defenses. The innate immune mediators also recruit and orchestrate an ensuing adaptive immune response, which acts in a pathogen-specific, adaptable manner, to effectively control the infection and develop long-term pathogen-specific memory.

Exerting pleiotropic effects on both innate and adaptive immune cells (such as macrophages, DCs, NK cells, B cells and T cells), vitamin D is implicated in regulating immune responses to bacterial and viral pathogens, cancer as well as in autoimmunity. The role of vitamin D in regulating innate antibacterial immunity is covered in Chapter 94 by Ismailova and White, and Chapter 96 focuses on regulation of adaptive immunity by vitamin D by Hawrylowicz et al. In this chapter, we first discuss epidemiological associations between vitamin D insufficiency and susceptibility to distinct viral infections and disease, followed by empirical and clinical evidence of innate and adaptive immune regulatory functions of vitamin D in the context of viral infections, and a consideration of potentially beneficial effects of vitamin D supplementation in mitigating clinical disease. In the following discussions, vitamin D sufficiency is generally defined as serum concentrations of 25(OH)D between 50 nmol/L (20 ng/mL) and 125 nmol/L (50 ng/mL) [32,33]. The complex virus–host interactions and immune factors that drive disease control or resolution are unique for distinct viruses. Hence, the antiviral immunomodulatory effects

of vitamin D are complex, and both unique virus-specific aspects as well as unifying principles are discussed here.

## 2. Association between vitamin D insufficiency and viral disease in epidemiological and interventional studies

Observational epidemiological and interventional studies provide a direct association between vitamin D insufficiency and susceptibility to several viral diseases such as acquired immunodeficiency syndrome (AIDS), hepatitis, dengue, herpes, respiratory diseases caused by influenza virus, respiratory syncytial virus (RSV), rhinovirus, and more recently coronavirus-induced disease 2019 (COVID-19), rotaviral gastrointestinal disease, and papillomavirus-induced genital warts and cervical cancer.

### 2.1 Acquired immunodeficiency syndrome

AIDS is caused by a lentivirus, human immunodeficiency virus (HIV), which is a single-stranded, positive-sense, enveloped RNA virus [34]. Poor vitamin D status (as assessed by serum levels of 25(OH)D or 1,25(OH) $_2$ D) is prevalent in HIV-infected patients and is associated with increased AIDS severity and risk of morbidity and short-term mortality [35–40]. Vitamin D insufficiency is associated with significantly higher viral loads in HIV/Kaposi's sarcoma patients, and reduced responsiveness to antiretroviral therapy [41,42]. Consistent with this, HIV-1-exposed seronegative individuals with natural resistance to HIV-1 infection exhibited high levels of 25(OH)D and its receptor (vitamin D receptor, VDR) [43]. Several VDR gene polymorphisms have also been associated with susceptibility to HIV infection and AIDS pathogenesis [44–46]. Moreover, supplementation with 25(OH)D has been shown in independent studies to significantly decrease viral loads and increase the number of circulating leukocytes including naïve CD4 $^+$  T helper (T $_H$ ) cells [47,48]. However, in contrast to the studies discussed before, there are reports of no association between 25(OH)D levels and disease complications [49] or antiretroviral therapeutic outcomes in HIV patients [50]. Likewise, three independent studies noted no significant changes in HIV viral loads, T cell counts, or mortality after supplementation with 25(OH)D or 1,25(OH) $_2$ D in HIV patients [51–54].

### 2.2 Viral hepatitis

Hepatitis C and B viruses are the main etiologic agents of viral hepatitis, an inflammatory liver disease caused by infection of hepatocytes [55,56].

Hepatitis C virus is a hepatotropic, single-stranded RNA virus, which causes inflammation that typically

progresses to liver cirrhosis and hepatocellular carcinoma (HCC) in case of chronic infection [55]. Vitamin D insufficiency (plasma 25(OH)D levels <20 ng/mL) is common among HCV patients, and an inverse correlation between vitamin D levels, viral loads, liver inflammation, fibrosis and cirrhosis hepatic encephalopathy, mortality rate, and progression to hepatocellular carcinoma have been noted in HCV patients [57–63]. Moreover, vitamin D insufficiency (25(OH)D) is associated with decreased response rate to older standard of care antiviral therapy (IFN- $\alpha$ +ribavirin) [57,64–69]. It is unclear whether lower vitamin D levels are a consequence of damaged livers or play a causal role in viral control. In contrast, supplementation with 25(OH)D has been shown to decrease liver fibrosis and increase sustained virological response (SVR) rate to antiviral therapy (IFN- $\alpha$ +ribavirin) [64,65,68,70–73]. Polymorphism of several vitamin D-related genes, such as VDR, vitamin D binding protein (DBP), cytochrome P450 family 24 subfamily A member 1 (CYP24A1) responsible for 24-hydroxylase enzymatic activity, and the 25-hydroxylase enzyme cytochrome P450 2R1 (CYP2R1), is implicated in HCV disease pathogenesis [74–76]. However, some studies have shown no effect of vitamin D insufficiency (circulating 25(OH)D) on viral growth, liver disease, SVR, or supplementation [77–85].

Hepatitis B virus, an enveloped DNA virus, also causes hepatitis. Acute hepatitis related to HBV infection can lead to liver failure and death, whereas viral chronicity is associated with long-term complications such as liver cirrhosis, hepatocellular carcinoma, and high rates of morbidity and mortality [56]. As in the case of HCV patients, epidemiological studies show significantly lower levels of circulating 25(OH)D in chronic HBV patients, with increased HBV DNA loads, decreased seroclearance, and clinical progression of liver cirrhosis outcomes [86–88]. SVR to telbivudine antiviral therapy was also decreased in patients with vitamin D insufficiency [89]. Consistent with this, transgenic murine model of HBV infection showed improved SVR to IFN- $\alpha$  therapy in combination with 25(OH)D supplementation [90]. VDR gene polymorphisms are also associated with distinct disease outcomes, and responses to IFN- $\alpha$  treatment [91–98]. However, several studies also reported contradictory results regarding the effects of vitamin D on HBV infection with no evident association between HBV DNA and serum 25(OH)D levels, liver inflammation, fibrosis, or treatment outcomes with tenofovir plus IFN- $\alpha$  combination therapy [89,99–102].

### 2.3 Herpes

Herpes is caused by human alphaherpesvirus 1 and human alphaherpesvirus 2, double-stranded DNA viruses, which typically remain latent in neurons, but

may undergo occasional reactivation to cause herpetic lesions in mouth, lips, nose, or genitals [103]. A significant association between low serum 25(OH)D levels and the presence of recurrent herpes labialis has been reported [104]. Treatment with 25(OH)D and 1,25(OH)<sub>2</sub>D inhibits HSV-1 growth in HeLa cells infected with HSV-1 [105]. Likewise, a recent report of amelioration of herpes simplex virus-induced Behçet's disease-like inflammation in mice by 1,25(OH)<sub>2</sub>D through downregulation of Toll-like receptors also argues in favor of beneficial effects of vitamin D signals during intracellular infections [106].

### 2.4 Human cytomegalovirus

HCMV, a beta-herpes double-stranded DNA virus (also referred to as human herpes virus 5, HHV5), has widespread seroprevalence worldwide [107,108]. While largely asymptomatic in immunocompetent hosts, acute HCMV infection causes significant morbidity and mortality in immune compromised individuals such as transplant recipients due to lytic viral replication and immune pathology. Lifelong viral persistence in normal hosts is associated with chronic inflammation and chronic allograft rejection. Vitamin D insufficiency has been associated with increased incidence of HCMV disease in infected individuals and increased opportunistic infections with HCMV in kidney transplant recipients [109–111]. However, in apparent contradiction, treatment with 1,25(OH)<sub>2</sub>D failed to inhibit HCMV replication in human foreskin fibroblasts infected with CMV possibly because of VDR downregulation postinfection [112].

### 2.5 Mononucleosis

Epstein–Barr virus (EBV), also known as human herpesvirus 4 (HHV4), a double-stranded DNA virus, is one of the most common human viruses that largely remains latent and causes symptoms mostly in immunocompromised situations [113]. Vitamin D insufficiency is associated with infectious mononucleosis and EBV infection [114].

### 2.6 Dengue fever

Dengue virus, a mosquito-borne, single positive-stranded RNA virus, causes dengue fever [115]. In the case of dengue infections, VDR gene polymorphisms have been identified as predictors of hemorrhagic fever and shock [116], and effects of 1,25(OH)<sub>2</sub>D supplementation on viral replication, immune response, and morbidity have been described [117,118]. High-dose vitamin D treatment also decreased dengue virus infection in patient monocyte-derived dendritic cells [119]. However, paradoxically, low serum 25(OH)D concentrations in dengue fever patients reduced the pathological



progress into dengue hemorrhagic fever/dengue shock syndrome, and circulating 25(OH)D was found to be much higher during an acute dengue episode than during disease-free periods [120].

## 2.7 Virus-induced cervical cancer

Human papilloma virus (HPV) is a small double-stranded circular DNA virus. With over 100 different types of HPV, majority of the infections resolve. However, infection with high-risk type HPV (HPV16 and HPV18) is responsible for the development of cervical cancers in women [121]. With very limited epidemiological studies on vitamin D and HPV infections, there is contrasting evidence both in support of an association between vitamin D insufficiency and HPV prevalence in sexually active women, and against any association between vitamin D insufficiency and the development of cervical cancer [122,123].

## 2.8 Viral diarrhea

Rotavirus is a double-stranded RNA virus that causes diarrheal disease [124]. Rotaviral diarrhea has been associated with vitamin D insufficiency [125]. Consistent with this, treatment with 25(OH)D has been shown to decrease rotavirus replication in intestinal enterocytes in vitro, and in vivo supplementation with 25(OH)D in pigs mitigated inflammation and intestinal damage during porcine epidemic diarrhea virus (PEDV) infection [126].

## 2.9 Pulmonary diseases

Several respiratory tract infections (RTI) such as influenza, respiratory syncytial virus (RSV), parainfluenza, adenovirus, rhinovirus, and related hospitalizations are associated with low serum 25(OH)D levels [127–142]. In contrast, higher 25(OH)D levels in the cord serum have been associated with reduced risk of multitriggered and virally induced wheezing in children [138]. A significant association between polymorphism of VDR, DBP, and the monohydroxylase cytochrome P450 3A4 (CYP3A4) and respiratory viral infections has also been observed [143–148]. Nonetheless, it is debated that these associations between vitamin D insufficiency and seasonal influenza incidences [149,150] might be related to decreased humidity and longevity of viral infectivity due to low temperatures than fluctuations in vitamin D [151,152]. Some studies have reported no effect of vitamin D insufficiency on severity of acute bronchiolitis caused by RSV or the burden of influenza virus infection [128,153–155]. Consistent with this, rhinovirus replication in primary human bronchial epithelial cells was unaffected by

1,25(OH)<sub>2</sub>D levels [156]. While several controlled vitamin D supplementation studies have been conducted in respiratory infections, the results were variable with some studies reporting beneficial effects, while others did not observe beneficial effects in influenza, RSV, or rhinovirus infections [157–159]. Some studies even reported increased frequency of infectious pneumonia [160]. Notwithstanding, a systematic review of randomized controlled trials from 1948 to 2009 has found strong evidence supporting beneficial effects of 1,25(OH)<sub>2</sub>D supplementation for treating acute respiratory illness, tuberculosis, and influenza [161].

## 2.10 COVID-19

Coronavirus-induced disease 2019 is caused by a coronavirus, SARS-CoV2, a positive-sense single-stranded RNA virus that has infected over 643M people and claimed over 6.63M lives in the global pandemic over the past 3 years. Several predisposing factors linked to exacerbated respiratory disease severity in COVID-19, such as age, genetic or ethnic backgrounds, geographical location, and preexistent chronic disease conditions [162–165], are also associated with inadequate vitamin D levels [166–170], thus supporting a possible indirect link between vitamin D insufficiency and COVID-19 severity [164,165,171,172]. There are several review articles that have discussed this concept [173–176].

Cursory cross-sectional global analyses have uncovered higher COVID-19-related mortality in countries with lower average vitamin D status [177]. In several retrospective studies, low levels of 25(OH)D were associated with increased infection, respiratory disease severity, or mortality in SARS-CoV2 infected patients in Sicilian, African American, Swiss, Iranian, and Saudi Arabian patients [178–182]. In a recent retrospective chart review analysis, patients aged  $\geq 65$  years showed a statistically significant association of vitamin D sufficiency with decreased odds of death, acute respiratory distress, and severe sepsis/septic shock, after adjustment for potential confounders [183]. Elderly COVID-19 patients also show increased pulmonary complications, longer sickness, and increased risk of mortality with vitamin D insufficiency compared with age-matched control subjects [184]. Likewise, significantly lower 25(OH)D levels were noted in severely symptomatic COVID-19 patients and were associated with prolonged mechanical ventilation, worse health evaluation score, COVID-19-induced acute respiratory distress syndrome (ARDS), and higher mortality [185,186]. A recent metaanalysis of multiple retrospective and prospective cohort, cross-sectional, case-control, and randomized controlled trial studies (up to November 26, 2020) investigating the relationship

between vitamin D status and SARS-CoV2 incidence or COVID-19 disease severity found a higher risk of SARS-CoV2 infection and COVID-19 severity in groups with compromised vitamin D status in studies that were adjusted and nonadjusted for confounders [163,187–189]. Studies with crude overall response (OR) and the adjusted Cox survival method also revealed a significant inverse association of mortality with 25(OH)D levels in Kazemi et al. [187] and another metaanalysis of studies from Europe and Asia [190]. Another metaanalysis found a trend, albeit statistically insignificant, toward inverse correlation between serum 25(OH)D levels and risk of mortality, ICU admission, and ventilation [191].

Studies of high-dose oral 25(OH)D supplementation (60,000 IU per day for at least 7 days) in SARS-CoV2 infections showed significantly lower rates of ICU admission and enhanced viral clearance and inflammation in a random controlled trial in India [192]. Three distinct British, Greek, and Italian studies of 25(OH)D supplementation in COVID-19 patients also showed significantly reduced risk of mortality [193–195]. However, a multicenter, double-blind, randomized, placebo-controlled trial in Brazil did not find beneficial effects of 25(OH)D supplementation (one dose of 200,000 IU) on the duration of hospital stay or need for mechanical ventilation, compared with the placebo control group [196]. The differences in these studies could be ascribed to differences in timing and dosage of supplementation and patient demographics.

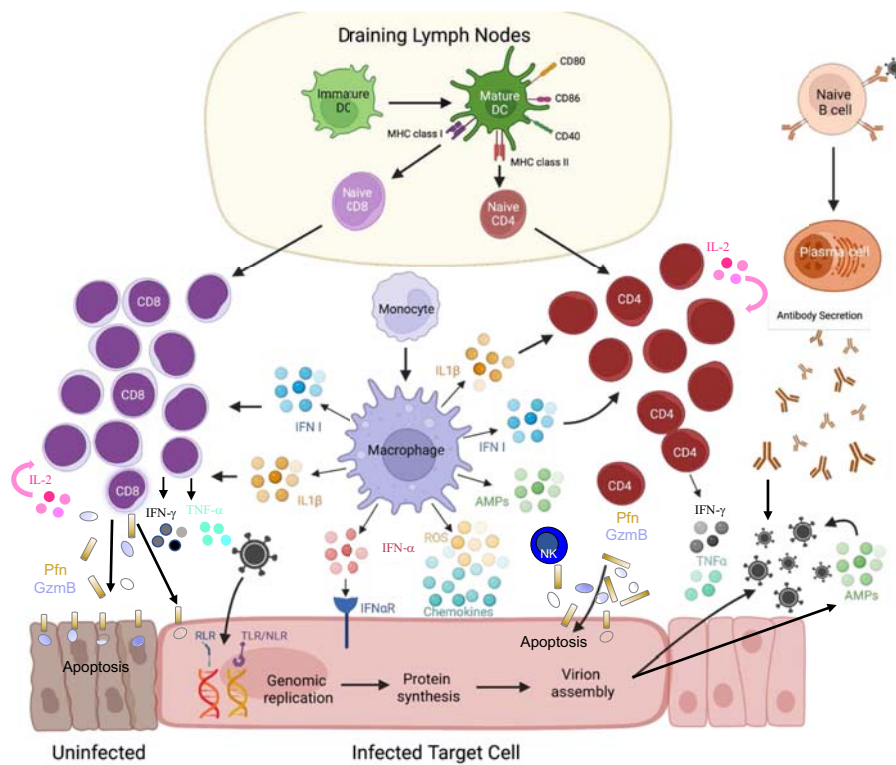
Overall, these epidemiological and interventional studies support a beneficial role of vitamin D supplementation against a variety of viral infections as well as COVID-19, particularly in the elderly, African Americans, and those with low vitamin D status. Nonetheless, contradictory data also abound for most viral infections [197]. This may be ascribed to differences in baseline levels of vitamin D, methods of assessment of vitamin D status, patient demographics, genetics, sample size, seasonal variations, timing and form of vitamin D supplementation (no universal consensus on biologically relevant doses of vitamin D for use in randomized controlled trials), and questionable effectiveness of supplementation [198] due to variation in the method of assay of serum 25(OH)D levels. Moreover, unique modes of viral replication, pathogenesis, and immune control may be impacted differentially by vitamin D signals in distinct infections. In the following sections, we will review the distinct virus-specific mechanisms by which vitamin D regulates disease outcomes in various infections and garner unifying principles of vitamin D regulation of antiviral immunity. The topic of vitamin D and COVID-19 is discussed in greater detail in Chapter 99.

### 3. Immunological mechanisms of viral control

Protective antiviral immune responses comprise concerted actions of the innate and adaptive arms of the immune system [29–31] (Fig. 95.1). As the first line of defense, the broadly reactive innate immune system is triggered by conserved pathogen-associated molecular motifs referred to as PAMPs and DAMPs released by infected target cells through PRRs. TLR signaling then leads to induction of antimicrobial peptides (AMPs). Activation of the innate immune system also results in induction of molecular mediators that directly counter viral growth by targeting key viral and cellular proteins. In addition, inflammatory antiviral cytokines and chemokines produced by innate immune cells serve to further amplify the immune response by recruiting additional innate and adaptive immune cells to the sites of infection. In case the innate immune system is ineffectual at controlling the pathogen, activation and expansion of pathogen-specific lymphocytes represents a targeted counterattack, where T cell and B cell clones against multiple pathogen-specific epitopes mount virus-specific humoral (antibody) and cellular (T cell) responses to eliminate the virus and virally infected target cells. In addition to adapting toward a higher affinity of interaction with viral epitopes, the adaptive immune system is also characterized by the ability to establish a long-lived immune memory compartment after clearance of the primary infection. Immune memory reacts more rapidly and vigorously to a pathogenic rechallenge, thus imparting protection from future reinfection. However, in certain cases of chronic viral infections where the virus persists (such as HIV, HCV), long-lived antigen-independent protective memory does not form, and T cells remain in a functionally repressed state of “exhaustion” [199].

### 4. Vitamin D and immunomodulation

The concept that vitamin D might exert immunomodulatory effects was first seeded about four decades ago by extrarenal production of bioactive vitamin D (1,25(OH)<sub>2</sub>D) by monocytes/macrophages [200–203]. This concept is further supported by findings of VDR and/or enzyme 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase) cytochrome P450 family 27 subfamily B member 1 (CYP27B1) expression by other immune cells such as T cells and B cells [166,204–207]. Vitamin D mediates its immunomodulatory effects through its bioactive form, 1,25(OH)<sub>2</sub>D, which binds to the vitamin D receptor (VDR), a nuclear receptor, thus leading to its dimerization with an isoform of the retinoid X receptor (RXR). The VDR-RXR heterodimers then activate transcription of genes by binding to vitamin D response



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**FIGURE 95.1 Overall schematic of immune network of cellular and molecular mediators involved in antiviral immunity.** Antiviral immune responses are typically triggered by innate sensing of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as RIG-I like receptors (RLRs), Toll-like receptors (TLRs), and NOD-like receptors (NLRs), which induce production of antimicrobial peptides (AMP), antiviral cytokines such as interferon- $\alpha$  (IFN- $\alpha$ ), and other proinflammatory cytokines such as IL-1 $\beta$ . Priming of CD4 and CD8 T cells by dendritic cells (DCs) leads to effector T cell responses, which include production of antiviral cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), as well as target cell killing through effector molecule perforin (Pfn) and serine protease granzyme B (GzmB). Natural killer cells (NKs) also mediate killing of infected target cells through GzmB and Pfn. Priming of B cells also leads to production of virus-specific antibodies, which mediate viral neutralization and antibody-dependent cell cytotoxicity (ADCC) of infected cells.

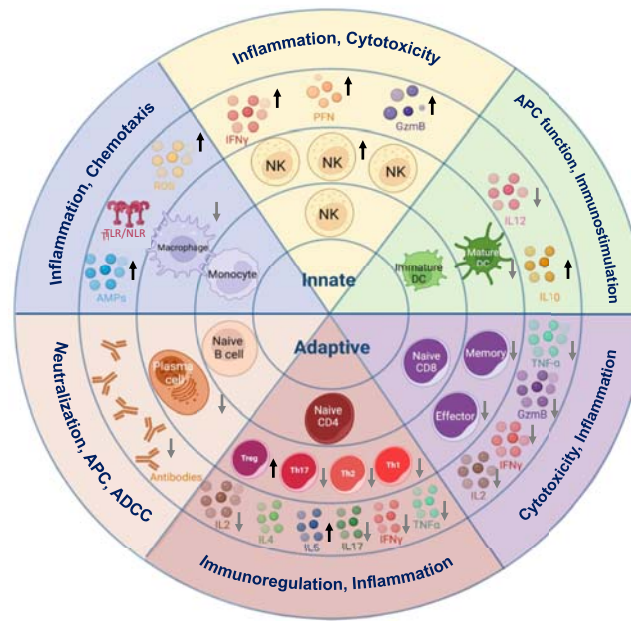
elements (VDREs) in the promoter regions or suppress gene transcription driven by nuclear factor of activated T cells (NFAT) by displacing it from the promoter regions [166,208–211]. Local production of 1,25(OH) $_2$ D by CYP27B1 in immune cells and sensing by VDR expressed on immune cells is proposed to modulate immune function in a paracrine and/or autocrine manner. Immunological outcomes of vitamin D signaling and downstream gene regulation include alterations in a variety of innate and adaptive immune functions (Fig. 95.2), such as regulation of monocyte/macrophage activation [212], induction of autophagy and apoptosis [213–215], increased production of AMPs [216–219], inhibition of inflammation [220–225], regulation of DC maturation, function, and antigen presentation [226–236], inhibition of B cell function [237–240], modulation of T cell trafficking, expansion, and effector functions, and induction of immune regulatory functions [241–250]. In the following sections, we discuss how these immune cells and processes are regulated by vitamin D in the context of viral infections potentially

impacted by host vitamin D status (see Section 3 of this chapter).

## 5. Innate immune sensing of viruses and 1,25(OH) $_2$ D

Toll-like receptors (TLRs) and retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLR) are PRRs, which trigger innate immune responses to viral infections through recognition of PAMPs in viral proteins and nucleic acids [29,251–253]. For example, endosomal TLR3 and TLR7, or cytosolic sensors retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) PRRs are implicated in triggering an innate immune response to SARS-CoV2 viral RNA [254]. Notably, double-stranded RNA, one of the key nucleic acid PAMPs from viral genomes has been shown to increase the expression of CYP27B1 in primary human lung epithelial cells, thus suggesting that PRR signals may upregulate vitamin D signaling [255].





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**FIGURE 95.2** Overview of immunomodulatory effects of 1,25(OH)<sub>2</sub>D on components of the innate and adaptive immune systems, typically involved in antiviral immunity. The physiologic outcomes are indicated by arrows indicating increase or decrease in the levels or functions of molecular and cellular mediators of immunity.

TLR1/TLR2 signaling in macrophages enhances CYP27B1 and VDR expression, thus resulting in an increase in localized levels of bioactive vitamin D [256]. In turn, vitamin D signals have been shown to modulate TLR4 signaling through regulation of CD-14 coreceptor expression [257,258]. In HIV-1 infection, TLR8 signals have been shown to inhibit viral growth in human macrophages through induction of VDR, CYP27B1, AMPs, and autophagy [259]. Furthermore, 25(OH)D or 1,25(OH)<sub>2</sub>D supplementation of HeLa cells was shown to significantly downregulate HSV-1 titers, albeit alongside downregulation of TLR2 and TLR9 mRNA levels, possibly due to postsignaling receptor downregulation [105]. Together, these data support the notion that PRR signaling by viral motifs may trigger the vitamin D pathway in infected target cells; vitamin D signals in turn might regulate PRR signaling, while contributing to viral control by augmenting production of AMPs and potentially blocking cellular processes that support viral growth.

## 6. Antimicrobial peptides in antiviral immunity and 1,25(OH)<sub>2</sub>D

AMPs or host defense peptides (HDPs) are molecular effectors of the innate immune response that exert potent, broad-spectrum antimicrobial functions against bacteria, enveloped viruses, fungi, and even cancer cells

through membrane perturbation, blocking of viral entry into host cells, enhanced activation of PRRs through viral proteins and nucleic acids, induction of inflammatory cytokine production, chemoattraction, or production of reactive oxygen species [28,260–266]. Produced by both immune cells (neutrophils, monocytes, NK cells, T cells and B cells [267]) and somatic cells (epithelial cells in the eye, respiratory tract, digestive tract, intestines, urinary tract, and skin) [218,268–270]), AMPs are secreted into the blood. AMPs are also released at the site of viral infection and immune function, where they may also exert chemotactic function to attract neutrophils, monocytes, and lymphocytes [31,271]. In the case of a bacterial respiratory infection, *Pseudomonas aeruginosa*, AMPs have been shown to promote pathogen clearance by inducing apoptosis of infected airway epithelial cells [272]. AMPs have also been shown to activate airway epithelial cells by transactivation of the epidermal growth factor receptor, thus offering potential new mechanisms of action for control of respiratory viral infections [273].

Vitamin D and CYP27B1 have been shown to induce the expression of AMPs such as human beta defensin 2 (HBD2) and cathelicidin [216,217,274,275]. In fact, in the absence of 1,25(OH)<sub>2</sub>D, VDR, or CYP27B1, macrophages and keratinocytes produce minimal cathelicidin [257]. Antiviral effects of AMPs, HBDs, and cathelicidin (or LL-37, the bioactive form of cathelicidin AMP) are well documented against influenza, HIV-1, HSV-1,



HPV, RSV, adenovirus, vaccinia virus, rhinovirus, and HCV [7,269,276–287]. In mouse model of influenza A infection, cathelicidin decreased viral replication and disease severity [288]. In the case of HIV-1, LL-37 inhibited viral reverse transcriptase activity [289]. Additionally, vitamin D–induced AMPs are proposed to be critical for natural resistance to HIV-1 based on positive correlation of elevated levels of VDR, cathelicidin, and human alpha defensin 4 (HAD-4) mRNA in PBMCs and oral mucosa of HIV-1-exposed seronegative individuals [43,290,291]. LL-37 also inhibits HSV-1 corneal infection by blocking virus binding to target host cells [292]. LL37, which is induced by vitamin D signals, has been shown to attenuate HCV infection and has been proposed for combination treatment with the traditional anti-IFN therapy [293]. Likewise, 1,25(OH)<sub>2</sub>D has been shown to attenuate rhinovirus [282,284,294,295] and rotavirus infection by expression of cathelicidin [8]. High concentrations of 1,25(OH)<sub>2</sub>D also increased AMPs in dengue viral infection [296]. Moreover, LL-37 and human  $\beta$ -defensin-2 (hBD2) were recently shown to bind directly to SARS-CoV-2 Spike protein, thus blocking binding to its receptor ACE2, and inhibiting viral entry [266,297]. Thus, vitamin D may enhance viral control through induction of AMPs, which block various stages of viral growth such as receptor binding, target cell entry, genomic amplification, viral enzymatic activity, and virion stability through protein–protein interaction and membrane destabilization.

## 7. Antiviral cytokine and chemokine responses and 1,25(OH)<sub>2</sub>D

PRR signaling typically culminates in activation of IRF-3 and IRF-7 transcription factors that induce production of type I interferons and numerous interferon-stimulated genes (ISGs). Other transcription factors, such as AP-1 family members, and NF- $\kappa$ B also participate in cytokine production during viral infections [28,251,298,299]. In addition to direct antiviral functions, inflammatory cytokines serve to amplify the antiviral immune responses by activating immune cells and promoting their migration, expansion, and effector functions at the sites of pathogen growth. However, overexuberant and/or prolonged inflammation may lead to immunopathology in healthy tissues. For example, excessive inflammation in the lung causes significant pulmonary damage [295], and aberrant inflammation is a lead cause of exacerbated lung disease in tuberculosis (TB) and flu [300]. In the highly pathogenic 1918 influenza A pandemic, pulmonary pathology and fatal outcome were associated with accelerated activation of proinflammatory genes that remained unabated until death [301–303]. Even in SARS-CoV2, increased

inflammation is the key underlying morbidity/mortality [174,304–308].

In general, inadequate vitamin D signals are associated with significantly higher inflammation in viral, bacterial, and autoimmune disorders [232,249,309,310]. In six of seven random control trials of vitamin D in highly inflammatory conditions (such as acute infantile congestive heart failure, multiple sclerosis, inflammatory bowel disease, cystic fibrosis, systemic lupus erythematosus [SLE], active TB, and evolving myocardial infarction), significant antiinflammatory effects were reported [311]. In the context of viral infections, inflammation and 25(OH)D levels were inversely related in influenza and COVID-19 patients as assessed by inverse association between disease severity and CRP levels, IL-6, and IFN- $\gamma$ , as well as a reciprocal relationship with T<sub>H1</sub> genes [185,304,312–314]. Nonetheless, a recent multicenter, double-blind, placebo-controlled, randomized clinical trial did not show improvements in inflammatory cytokines such as IL-12, IL-17, IFN- $\gamma$ , granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-8, interferon-inducible protein-10 (IP-10), macrophage inflammatory protein-1 *beta* (MIP-1 $\beta$ ), monocyte chemoattractant protein-1 (MCP-1), or growth factor vascular endothelial (VEGF) in hospitalized patients with moderate to severe COVID-19 compared with placebo following a single dose of 200,000 IU vitamin D<sub>3</sub> [315].

Largely, anti-inflammatory effects of vitamin D are noted in influenza infection. Administration of high-dose 1,25(OH)<sub>2</sub>D in mouse model of influenza virus infection reduced viral replication as well as inflammatory cytokines, IL-5, IL-6, IL-8, TNF- $\alpha$ , and IFN- $\gamma$  [316]. In addition, latent HIV-1 reactivation is also inhibited by vitamin D through reduction of TNF- $\alpha$  [317]. Likewise, in RSV infection, 1,25(OH)<sub>2</sub>D reduced the expression of NF- $\kappa$ B- and STAT1-driven proinflammatory gene expression programs [137,318]. In rotavirus infection as well, 1,25(OH)<sub>2</sub>D exerted an immunosuppressive role through the miRNA-155-5p-mediated regulation of IRF3 signaling [319]. In the case of dengue virus infection, concentration-dependent immunomodulatory effects of 1,25(OH)<sub>2</sub>D were seen in *in vitro*–infected cells, with immunostimulatory effects of higher concentrations on both proinflammatory (interferon stimulated gene 15, ISG15 and IL-12p70) and anti-inflammatory cytokines (IL-10), and immunosuppressive effects at lower concentrations [15,296,320]. The TLR4/NF- $\kappa$ B/miR-155–5p/suppressor of cytokine signaling (SOCS)–1 axis is involved in this process [321]. Whether physiologic levels of bioactive vitamin D in anatomic micro-niches induce proinflammatory or antiinflammatory immune responses in dengue virus infection remains to be determined.

In contrast, vitamin D largely induced proinflammatory responses in HCV infection by enhancing the IFN

signaling pathway, STAT-1-mediated IFN- $\alpha$  production, and downregulating the TGF- $\beta$ 1/small mothers against decapentaplegic 3 (SMAD3) signaling axis [1,6,322,323]. The inflammatory chemokines/receptors regulated by 1,25(OH)<sub>2</sub>D include RANTES in influenza infection [316] and CCR5 in HIV-1 infection [13]. In contrast, CXCL8 and CXCL-10 are induced by 1,25(OH)<sub>2</sub>D in rhinovirus infection [156]. These complex disease-specific and dose-dependent effects of vitamin D on inflammation in distinct viral infection contexts may be interpreted as a largely balancing act to drive adequate inflammation and downstream immune response (such as lymphocyte activation, expansion, and effector differentiation) for achieving efficacious viral control, while mitigating deleterious immune pathology.

## 8. Natural killer cell responses and 1,25(OH)<sub>2</sub>D

Natural killer (NK) cells play an important role in innate immune responses against viral infections such as influenza A, HIV, and HCV. NK cells mediate direct cytotoxicity against virally infected target cells by recognizing and responding to virus-induced MHC-I downregulation or alterations in activating and inhibitory signals on infected host cells [324,325]. NK cells also promote target cell clearance and modulate adaptive immune responses through production of effector cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , and through antibody-dependent cell cytotoxicity ADCC [324,325].

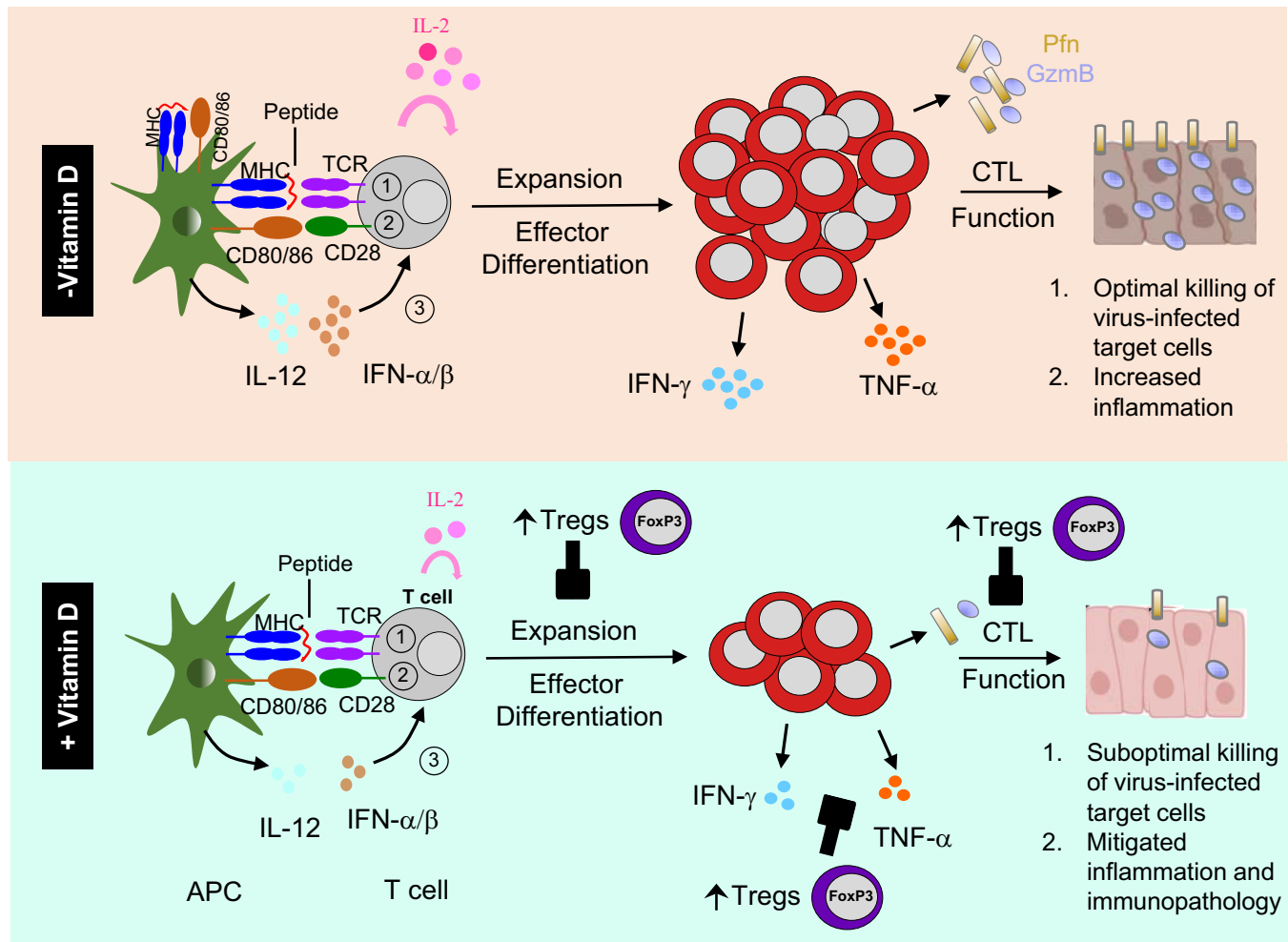
Vitamin D largely exerts a stimulatory role in NK cell responses, as suggested by increased NK cell degranulation and cytotoxic function in vitro through increased expression of effector molecules such as IFN- $\gamma$ , granzymes A and B, and degranulation marker lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) [326–331]. In vivo as well, direct associations between NK cell function and 1,25-(OH)<sub>2</sub>D levels have been reported [332–334], albeit some studies did not find a clear association [335,336], or even suggested an immunoregulatory role of vitamin D in NK cells [337,338]. In the context of viral diseases, particularly COVID-19, several studies have demonstrated an inverse association between 25(OH)D and NK cell numbers and function [339–342]. These discrepancies may be related to the altered immunologic factors during viral disease, which are not recapitulated in in vitro systems. With such limited datasets, it is imperative that further studies correlating vitamin D levels and NK cell quantity and function in patients infected with different viruses and cause–effect studies in preclinical disease models are conducted to garner better insight into vitamin D–dependent mechanisms of viral control through modulation of NK cells.

## 9. Antiviral CD4 T cell immunity and 1,25(OH)<sub>2</sub>D

CD4 T cells contribute to antiviral immunity by mediating direct cytotoxicity [343], and by producing antiviral and immunomodulatory cytokines, which further regulate CD8 and CD4 T cell effector responses as well as B cell responses [344]. CD4 T cell responses are initiated by presentation of cognate antigen in the context of MHC-II (signal 1), along with costimulatory (signal 2) and cytokine signals (signal 3) by APCs (primarily DCs), leading to clonal expansion and effector differentiation (Fig. 95.3). CD4 T cells differentiate into distinct subsets of effector lineages with unique functions depending on contextual cytokine signals during activation [345]. Antigenic stimulation of naïve CD4 T cells in the presence of IL-2, IL-12, and IFN- $\gamma$  drives the differentiation of proinflammatory T helper subset 1 (T<sub>H1</sub>) cells for efficacious defense against intracellular pathogens. Combination of IL-6, IL-21, IL-23, and TGF- $\beta$  cytokine signals drives the development of proinflammatory T helper 17 (T<sub>H17</sub>) T cells, also important in antiviral immunity [345,346]. IL-6 and IL-21 along with costimulatory ICOS signals and TCR signals, drive T follicular helper (TF<sub>H</sub>) cells, which help counter viruses by promoting virus-specific B cell immunity through B cell activation and antibody affinity maturation. A subset of CD4 T cells, regulatory T cells (Treg), which are stimulated by antigen, IL-2 and TGF- $\beta$  cytokines, and CD28 costimulatory signals, plays a crucial role in antiviral immunity by regulating T cell priming and effector functions of both CD4 and CD8 effector T cells [345].

Both CD4 and CD8 T cells express VDR and Cyp27B1, which is upregulated upon T cell activation [166,199], thus supporting the notion that T cells can respond to and produce bioactive vitamin D signals [347]. 1,25(OH)<sub>2</sub>D inhibits CD4 T cell activation and proliferation [348–350], possibly through repression of IL-2 [351]. 1,25(OH)<sub>2</sub>D signals interface with MAPK/Erk signaling pathway in many immune cell types and may regulate T cell activation and proliferation by modulating MAPK signaling [352–357]. In nonviral systems, trafficking of T<sub>H</sub> cells to tissue sites is regulated by vitamin D [246]. Additionally, 1,25(OH)<sub>2</sub>D increases proportions of Treg cells and reduces T<sub>H1</sub> and T<sub>H17</sub> cells [244,358–360]. Consistent with suppression of T<sub>H1</sub> differentiation by vitamin D, VDR expression is reduced on T<sub>H1</sub> cells [242]. Proinflammatory cytokines produced by T cells, such as IFN- $\gamma$ , TNF- $\alpha$ , and IL-2, induce Cyp27b1 expression [200,361–363], thus suggesting a possible negative feedback loop for regulating T cell responses.

Importantly, in the context of viral infections, 1,25(OH)<sub>2</sub>D treatment has been shown to reduce HIV-1



**FIGURE 95.3 Regulation of antiviral T cell immunity by 1,25(OH)<sub>2</sub>D.** Antiviral T cell responses in conditions of suboptimal (– Vitamin D; peach box) or increased (+ Vitamin D; teal box) vitamin D signals are presented. Increased maturation (as indicated by increased expression of MHC (signal 1), costimulatory molecules (signal 2), and inflammatory cytokines (signals 3) of antigen-presenting cells (APCs) is proposed to lead to increased activation and effector T cell responses under vitamin D insufficiency/deficiency conditions, while also inducing inflammation and immunopathology. On the other hand, vitamin D sufficiency conditions are proposed to moderate APC function, T cell activation, and effector functions in part through reduced production of proinflammatory (signal 3) cytokines, growth cytokine interleukin-2 (IL-2), and increased levels of Treg cells. This leads to regulated inflammation and immunopathology, with slightly impaired killing of virus-infected target cells by cytotoxic T lymphocyte (CTL) activity.

transmission by specifically modulating the activation of T cells [364]. Furthermore, consistent with largely suppressive effects of 25(OH)<sub>2</sub>D supplementation on proinflammatory cytokines such as IL-5 and IFN-γ in influenza A virus and SARS-CoV2 infections [185,304,312,313], expression of VDR and CYP27B1 was associated with transition from proinflammatory interferon-γ<sup>+</sup> T<sub>H</sub>1 cells to suppressive interleukin-10-producing cells upon complement activation [314]. Importantly, patients with severe COVID-19 showed a reciprocal relationship between T<sub>H</sub>1 and vitamin D–repressed gene signatures in bronchoalveolar lavage, thus indicating possible localized immunopathology, and supporting potential clinical benefit from vitamin D supplementation [314].

These immunosuppressive effects of vitamin D on T cells may be a combined result of autocrine and/or paracrine 1,25(OH)<sub>2</sub>D signaling in T cells, or through indirect effects of vitamin D on APCs. 1,25(OH)<sub>2</sub>D directly regulates the maturation and antigen presentation functions of monocytes/macrophages and DCs by downregulating surface expression of MHC class II and costimulatory molecules (such as CD40, CD80, and CD86) [212,365]. Vitamin D further drives differentiation of tolerogenic DCs with reduced IL-12 and TNF-α production and increased production of antiinflammatory IL-10, thus leading to preferential differentiation of Treg cells than inflammatory T<sub>H</sub> subsets [226–235,366,367]. These findings support a model of vitamin D–dependent modulation of T<sub>H</sub> fate

determination through alterations in the maturation and function of APCs. Further confirmations of vitamin D-dependent *in vivo* induction of tolerogenic DCs and skewing of T<sub>H</sub> responses in favor of Treg cells and against inflammatory T<sub>H1</sub> and T<sub>H17</sub> cells in multiple viral infections would solidify a universal role of vitamin D in regulating inflammatory antiviral T<sub>H</sub> cell responses through DC modulation.

## 10. Antiviral CD8 T cell immunity and 1,25(OH)<sub>2</sub>D

CD8 T cell immunity plays a crucial role in the control of viral infections including HIV-1, dengue virus, HBV, HCV, HSV, etc. [166,199]. CD8 T cells mount an antigen-specific response against virally infected target cells, which includes targeted killing of infected host cells through induction of apoptosis by perforin and granzyme B cytotoxic mediators or death receptor-mediated killing through Fas. CD8 T cells also produce cytokines, which exert antiviral effects, and/or immunomodulatory functions [28,368,369]. Naïve antigen-specific CD8 T cells are stimulated through the T cell receptor (TCR) by cognate peptide–MHC-I complexes on APCs in the secondary lymphoid organs. Costimulatory signals through CD28 are a necessary second signal for optimal activation [368,370]. Inflammatory cytokines, such as IL-12 and type-I interferons, serve as third signals for optimal expansion and effector differentiation of naïve CD8 T cells into cytotoxic T lymphocytes (CTLs) [370–376]. Fully differentiated effector T cells downregulate the expression of L-selectin and C–C chemokine receptor type 7 (CCR-7) and acquire the ability to migrate out of the lymph nodes, into peripheral sites of infection where they mediate effector functions such as specific lysis of infected targets and cytokine production.

As opposed to clear antiproliferative functions of vitamin D in CD4 T cells, 1,25(OH)<sub>2</sub>D has varying effects on CTL proliferation depending on the stimulation used [377–379]. With respect to effector CTL function, a study reported that pretreatment with 1,25(OH)<sub>2</sub>D compromises target cell lysis as assessed by Cr<sup>51</sup> release assay in the setting of an *in vitro* mixed lymphocyte reaction [380]. *In vitro* stimulation of CD8 T cells in the presence of higher concentrations of 1,25(OH)<sub>2</sub>D leads to reduced IL-2 and IL-17 [166,246,379,381,382]. Suppression of IL-2 (a key T cell growth factor) by 1,25(OH)<sub>2</sub>D is proposed as a potential mechanism for inhibiting T cell proliferation [351]. CD200, an immunoglobulin-like molecule that dampens proinflammatory activity of innate cells, is upregulated in human peripheral and airway resident CD8 T cells in response to 1,25(OH)<sub>2</sub>D [383].

In the context of human disease, 1,25(OH)<sub>2</sub>D has been shown to suppress production of effector cytokines (IFN- $\gamma$  and TNF- $\alpha$ ) by EBV-specific CD8 T cells, but enhance production of IL-5 and transforming growth factor (TGF)- $\beta$  in *in vitro* cytokine production assays [384]. In the well-established murine model of acute infection with lymphocytic choriomeningitis virus (LCMV), which is widely used to study antiviral effector and memory CD8 T cell responses, and was originally employed to establish the key immunological concept of MHC-I restriction of CD8 T cell responses [385,386], VDR deficiency was associated with increased CTL expansion, compromised effector differentiation and survival, dysregulated CTL homing, and development of a less diverse virus-specific CTL repertoire postactivation [382].

In summary, analogous to immunosuppressive effects in CD4 T cells, the limited data in CD8 T cells support a similar inhibitory role of vitamin D signals in CD8 T cell antiviral responses with respect to antigen-specific clonal expansion, effector CTL differentiation, diversity of effector and memory repertoire, and modulation of migratory and survival properties. However, how well *in vitro* observations reflect *in vivo* immunological outcomes of autocrine and paracrine vitamin D signals in CD8 T cells the context of diverse viral infections with complex milieu due to unique host–pathogen interactions remain to be fully addressed. In addition, it remains unclear whether vitamin D regulates CD8 T cell responses through direct signaling in CD8 T cells, or indirectly through modulation of APCs (such as DCs, macrophages, B cells). Likewise, the key transcriptional and metabolic mediators of vitamin D-dependent regulation of antiviral T cell immunity also remain to be elucidated.

## 11. B cell responses to viruses and 1,25(OH)<sub>2</sub>D

B cells mount an antiviral response through production of virus-specific antibodies, which serve as effector molecules by neutralizing viral infectivity and through antibody-dependent cell-mediated cytotoxicity (ADCC) [28]. B cells express both VDR and CYP27B1, which are further upregulated upon B cell stimulation through the B cell receptor on the cell surface [240]. Stimulation of naïve B cells through the BCR typically promotes their differentiation into effector plasma cell clones, which secrete high levels of antibodies of a given antigen specificity [369,387,388]. In addition, activated B cells also produce cytokines and participate in antigen presentation to T cells. In *in vitro* studies, 1,25(OH)<sub>2</sub>D inhibits differentiation of B cells into plasma cells and antibody production [237,240,241]. Inverse association between serum 25(OH)D levels and anti-EBNA-1 IgG titers has



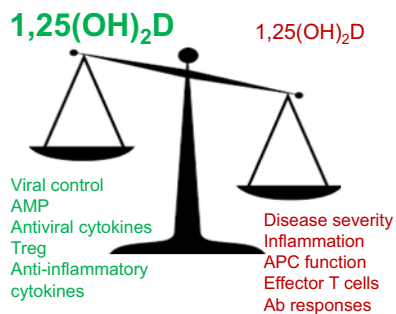
been reported prior to clinical MS disease manifestation, and 25(OH)D supplementation significantly reduced anti-EBNA-1 IgG titers [389]. Likewise, EBV-specific memory B cell formation is also inhibited by 25(OH)D [390]. However, another independent study did not find any changes in anti-EBNA-1 IgG titers upon supplementation [391]. On the other extreme, higher vitamin D levels were found to correlate with increased production of influenza virus–specific antibodies [158]. These findings underscore the importance of more in-depth cause–effect type studies on how B cell responses are modulated by vitamin D in the context of distinct viral infections.

## 12. Conclusions

Overall, 1,25(OH)<sub>2</sub>D signaling seems to play a key immune balancing act by promoting viral control through increased innate sensing of viruses, and enhanced production of AMPs and antiviral cytokines, on one hand (Fig. 95.4). On the other hand, 1,25(OH)<sub>2</sub>D suppresses deleterious bystander immunopathology by inhibiting the production of proinflammatory cytokines and chemokines, and enhancing the expression of antiinflammatory cytokines in certain viral infections (such as influenza, SARS-CoV2, and HIV-1). Consistent with this, inflammatory CD4 T<sub>H1</sub> and effector CD8 T cell responses are regulated, whereas regulatory CD4 T cells are enhanced by 1,25(OH)<sub>2</sub>D signaling in certain viral infections. Vitamin D–dependent alterations in immune cell trafficking to sites of viral infection and/or secondary lymphoid sites of immune activation are implied by observations of altered chemokine expression under conditions of varying 1,25(OH)<sub>2</sub>D signaling in viral infections. Nonetheless, our understanding of the

immunomodulatory functions of vitamin D in the context of different viral infections remains incomplete, especially as pertains to NK cell immunity and adaptive virus-specific T and B cell responses.

In general, 1,25(OH)<sub>2</sub>D signaling impairs maturation and antigen-presenting functions of macrophages, DCs, and B cells, thus offering regulation of antigen presentation as a potential mechanism of impaired T cell immunity in virus infections. Direct regulation of activation, expansion, and effector differentiation of virus-specific T cells and B cells by 1,25(OH)<sub>2</sub>D signaling is also implicated in vitamin D–dependent control of adaptive immune responses in viral infections. However, clear establishment of cause–effect relationship between 1,25(OH)<sub>2</sub>D signaling and modulation of antiviral immunity requires systematic and rigorous foundational preclinical studies of diverse viral infections in immunocompetent animal models, with an immunologically targeted enquiry into virus-specific mechanisms of immune control under tightly controlled in vivo conditions of vitamin D sufficiency, insufficiency, deficiency, and supplementation. Since viral infections largely occur through mucosal surfaces, how 1,25(OH)<sub>2</sub>D signaling impacts antiviral immunity at mucosal barriers is an important area of investigation that may be readily conducted in preclinical animal infection models. In parallel with preclinical studies, clinical correlations of vitamin D sufficiency or insufficiency with immune parameters of viral control will guide better informed randomized controlled trials of 1,25(OH)<sub>2</sub>D supplementation with tightly defined patient cohorts (with respect to variables such as age, race, ethnicity, genetics, weight, disease status at the time of enrollment), precise and universally accepted pre- and post-treatment measures of vitamin D metabolites, and virus-specific immune biomarkers of disease control. Collectively, these research findings will bolster generalized efforts of vitamin D supplementation at a population level to better manage viral outbreaks and epidemics in the future.



**FIGURE 95.4 Summary of the immunobalancing role of vitamin D in viral infections.** The existing data supports a model of immunoregulation by 1,25(OH)<sub>2</sub>D, where overall viral control (*green*) is enhanced through induction of innate antiviral immunity (such as production of AMPs and possibly NK cell function), whereas adaptive T cell immunity and proinflammatory immune responses (*red*) are regulated to possibly mitigate bystander immunopathology.

## 13. Summary points

- Preclinical and clinical reports suggest an association between circulating 25(OH)D levels and vulnerability to viral infections and disease severity in disease conditions such as AIDS, COVID-19, influenza, and other viral respiratory diseases, hepatitis, herpes, and viral diarrhea.
- 1,25(OH)<sub>2</sub>D signaling affects susceptibility to and severity of viral diseases via innate sensing of viruses through PRRs, induction of AMPs, and cytokines and chemokines in infected target cells and innate immune cells.

- In most viral infections, such as influenza, HIV, and SARS-CoV2, 1,25(OH)<sub>2</sub>D promotes balanced anti-inflammatory and proinflammatory cytokine responses to balance beneficial antiviral immunity and deleterious immunopathology.
- 1,25(OH)<sub>2</sub>D signaling largely favors regulatory CD4 T cell responses and suppresses inflammatory effector CD8 and CD4 T<sub>H1</sub>-type responses in viral infections through regulation of T cell activation, expansion, effector functions, and by regulating the immunostimulatory capacity of APCs.
- More directed clinical and preclinical studies are needed to build a complete picture of mechanisms underlying vitamin D-dependent regulation of antiviral NK cell, T cell, and B cell responses in distinct viral infections.

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## Further reading

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# Vitamin D and adaptive immunity in health and disease

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## OBJECTIVES

- Describe vitamin D actions on effector and regulatory T cell responses through direct effects on naïve and memory T cells and indirect effects via antigen-presenting cells.
- Review data on modulation of B cell and antibody responses, including responses to vaccination.
- Overview clinical studies of vitamin D status or repletion, where these support in vitro findings and potential explanations for disparities in clinical versus experimental studies.
- Discuss translational studies that support a role for vitamin D in maintaining immune homeostasis at the level of adaptive immunity, highlighting studies in airway disease.

## 1. Introduction

The immune system comprises both innate and adaptive arms. The former represents the first line of defense to pathogens such as bacteria (see [Chapter 94](#)) and viruses (see [Chapter 95](#)), through the actions of physical barriers (e.g., skin, bronchial epithelial cells), soluble

mediators (e.g., mucus, complement), and specialized immune cells (e.g., macrophages, neutrophils). These cells express pathogen recognition receptors (PRRs), such as the Toll-like receptor family (TLRs), which recognize pathogen-associated molecular patterns (PAMPs). Dendritic cells are innate cells that express many PRRs, reside at sites of pathogen entry, and form a crucial link between innate and adaptive immunity in their role as antigen-presenting cells (APCs) (see [Chapter 9](#)). It is therefore difficult to investigate the effects of vitamin D upon adaptive immunity, without also considering the effects upon innate immune cells, such as dendritic cells (see [Chapter 9](#)). Adaptive or acquired immunity is characterized by the generation over time of highly specific, long-lived immune responses that are required for protection against pathogen infection. A key characteristic of adaptive immunity is the capacity to respond more effectively and rapidly to antigens that have been previously encountered, which is termed immune memory and forms the basis of successful vaccination.

The key players of humoral and cell-mediated adaptive immunity are antibody-producing B lymphocytes and T lymphocytes, respectively. The latter contains two major categories, CD4 + and CD8 + T cells, which are further divided into subsets based on function. T and B cells respond not only to pathogens or components of pathogens but also to harmless antigens present

in our environment such as allergens. Inappropriate adaptive immune responses to self-antigens can lead to autoimmune conditions such as rheumatoid arthritis or multiple sclerosis. The failure to mount appropriate adaptive immune responses to tumor cells is also detrimental for the host. Parallel regulatory mechanisms have evolved to limit over-exuberant immune responses to pathogens, prevent damaging or inappropriate adaptive immune responses to self and environmental antigens, and thereby maintain immune homeostasis. Many diseases are characterized by a failure in adaptive regulatory responses, leading to loss of self-tolerance and the failure to control inappropriate immune responses.

The different classes of lymphocytes are programmed to provide finely tailored responses to diverse types of immune challenge. A crucial difference between B and T lymphocyte responses is that B cells directly recognize conformational determinants within antigens by a specific B cell antigen receptor (BCR or immunoglobulin), whereas T cells use their T cell receptor (TCR) to recognize peptide fragments of antigen bound to MHC class I (CD8) or MHC class II (CD4) on the surface of APCs. A commonality is that both the BCR and TCR exhibit immense diversity in their antigen binding sites enabling them to recognize a wide array of antigens.

Epidemiological evidence that links vitamin D status with the incidence and control of a wide range of disease conditions has garnered significant interest in not only whether restoring vitamin D sufficiency can improve health outcomes, but also the immune mechanisms modulated by vitamin D that underpin such effects. Vitamin D has well-documented effects on multiple, functionally divergent, immune effector, and regulatory pathways within the adaptive immune response, which forms the focus of this chapter in the context of human immune homeostasis and disease.

## 2. Epidemiological and other evidence supporting a role of vitamin D in immune disease

Two important lines of evidence support a role for vitamin D in control of adaptive immunity and immune-mediated diseases. Firstly, as covered throughout this series of reviews, many immune-mediated diseases demonstrate striking associations with latitude and/or levels of sun exposure, frequently used as surrogate indicators of vitamin D status, as well as directly with vitamin D status, commonly measured as circulating levels of the vitamin D precursor, 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>). Associations with multiple autoimmune conditions include with type 1 diabetes, multiple sclerosis, and inflammatory bowel disease. Associations between vitamin D insufficiency and deficiency and the incidence and severity

of disease have also been described for cardiovascular conditions, allergy, and cancers [1–3]. Associations with the incidence, severity, and treatment response in chronic respiratory disease, reflecting the authors' interests, are also prominent and therefore highlighted throughout this chapter [4,5] (see also Chapter 44).

Secondly, the vitamin D receptor (VDR) is expressed by essentially all immune cells either constitutively or following activation. Enzymes in the metabolic cascade leading to the generation of active vitamin D (1,25-dihydroxyvitamin D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub>) are expressed in tissues where adaptive immune responses occur, by structural cells such as barrier epithelial cells in the airways, skin keratinocytes, and innate immune cell populations such as dendritic cells and macrophages (see Chapter 9). T cells are dependent for their activation on signals from APCs or accessory cells such as dendritic cells and macrophages. In a two-way process, T cells and their products regulate the function of dendritic cells and macrophages. T cell-derived cytokines such as IL-4 and IFN-γ can modulate not only the antigen-presenting function of these cells, but also the bioavailability of 1,25(OH)<sub>2</sub>D<sub>3</sub> by regulating the expression of crucial enzymes in the metabolic cascade such as 1α-OHase (CYP27B1) and 24-OHase (CYP24A1) [6,7].

## 3. Translational studies as a window to study effects of vitamin D on adaptive immunology

The following sections describe laboratory observations and to a lesser extent reports in animal models of the effects of vitamin D on adaptive immune cell populations. However, to better understand physiologically relevant effects of vitamin D on adaptive immunity, one approach is to investigate how parameters identified to be of interest are linked to vitamin D status (serum levels of 25(OH)D) in patients, and less commonly in healthy individuals. A second approach is to study individuals pre and post-vitamin D supplementation or, rarely, following UVB irradiation. However, clinical study and trial designs have varied widely, making comparisons and clear conclusions challenging. Areas in which such studies vary include 25(OH)D status at the start of the study—which in some cases was unknown, or where participants were not shown to be 25(OH)D deficient/insufficient at the start of the trial. The dose and form of vitamin D (e.g., vitamin D<sub>2</sub> vs. vitamin D<sub>3</sub>), supplementation frequency (e.g., ranging from daily to 2 monthly bolus doses), and duration (a single bolus vs. daily/weekly/monthly delivery over days to months) of administration have all varied between different studies. Recent studies have also shown that serum 25(OH)D<sub>3</sub> levels, the most commonly used measure of vitamin D status, can decrease with inflammatory status (e.g., during acute-phase responses,

sepsis, and with increased bactericidal activity)—possibly suggesting that higher supplementation doses are required in these instances [8]. Furthermore, there is increasing awareness that ethnicity and polymorphisms in genes within the vitamin pathway can impact efficacy of repletion and nature of changes that occur [9]. As discussed in the following, vitamin D sufficiency is likely to promote immune homeostasis through dampening adaptive effector responses and promoting mechanisms of peripheral tolerance, in addition to important effects on innate and structural cells discussed elsewhere. Therefore, maintaining long-term vitamin D sufficiency is likely to be crucial and is an additional and important consideration in the interpretation of human supplementation studies. Examples of where vitamin D-mediated effects on adaptive immunity are mirrored through such translational approaches are highlighted throughout the following sections.

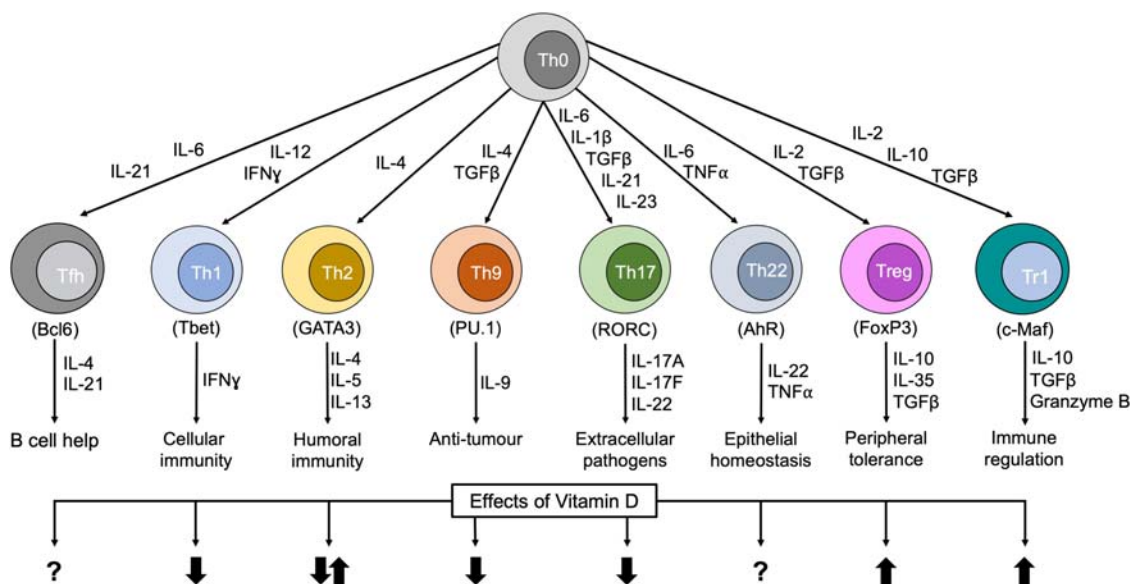
#### 4. Actions of vitamin D on proinflammatory T lymphocyte responses

A pioneering study in 2013 identified thousands of VDR-binding sites in the genome of T lymphocytes, suggesting that vitamin D has the capacity to control gene transcription in these cells [10]. Further studies identified VDR upregulation and expression of CYP27B1, required for conversion of  $25(\text{OH})\text{D}_3$  into  $1,25(\text{OH})_2\text{D}_3$ ,

in both CD4 and CD8 T lymphocytes following activation [11,12]. However, it is unclear what the physiological contribution of endogenous production of active vitamin D by human T cells is, with CD4 + T cells expressing CYP27B1 at comparatively low levels to dendritic cells, and conflicting reports on whether precursor  $25(\text{OH})_2\text{D}_3$  is able [13] or unable [14,15] to alter T lymphocyte responses without APCs also present in different experimental systems. Combined  $1,25(\text{OH})_2\text{D}_3$  from dendritic cells, accessory cells, and structural cells within the local milieu are likely to contribute to immuno-modulatory effects on T cells, as well as autocrine effects following T cell activation (see Chapter 9 for further discussion of local vs. systemic synthesis of  $1,25(\text{OH})_2\text{D}_3$ ).

#### 5. Naïve T cells

Studies on the effects of vitamin D on T lymphocytes have focused on the control of previously activated or memory cells, and effector and regulatory T cell function, with fewer reports of how vitamin D influences naïve T cells. Naïve T cells are critically dependent on dendritic cells for activation and signals that influence their differentiation into functionally distinct subsets (Fig. 96.1). By maintaining dendritic cells in an immature or tolerogenic state, vitamin D is more likely to



**FIGURE 96.1 CD4<sup>+</sup> T helper cell subset differentiation.** Naïve CD4<sup>+</sup> T cells differentiate, upon antigen encounter, into distinct subsets influenced by signals from antigen-presenting cells and the local inflammatory milieu. These subsets are characterized by their master transcription factors (in brackets) and cytokine profiles. Vitamin D has varying effects upon CD4<sup>+</sup> T cell subsets, decreasing Th1 and Th17 responses, while increasing IL-10 expression and Foxp3<sup>+</sup>Tregs, with more complex reports upon Th2 immunity. This is likely to reflect both indirect effects of vitamin D upon antigen-presenting cells and direct effects on T cells. T effector cells exhibit plasticity and can co-express their signature cytokine, such as IFNγ or IL-17A, with other pro-inflammatory cytokines, or anti-inflammatory cytokines such as IL-10 in a process modulated by vitamin D in favor of co-expression of IL-10.



favor naïve T cell maintenance and the generation and maintenance of regulatory T cells.

Naïve T cells express no or low levels of VDR and require activation to increase VDR [16]. In studies in mice, vitamin D was reported to activate naïve CD4+ T cells, albeit following culture in the absence of APCs for 1–3 weeks, to promote a T helper (Th)2 response and upregulate the Th2-associated transcription factors c-Maf and GATA3, while downregulating Th1 responses [17]. A second study suggested 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibited both Th1 and Th2 responses when added at the start of *in vitro* polarization in naïve T cell cultures; however, if cells were first activated and 1,25(OH)<sub>2</sub>D<sub>3</sub> added after 4–7 days of culture, then Th2 responses were no longer reduced [18]. Using human cells, von Essen demonstrated that VDR expression by naïve T cells was induced by TCR signaling via the alternative mitogen-activated protein kinase p38 pathway. TCR signaling via p38 was proposed to drive successive induction of VDR and PLC-γ1, which were required for subsequent classical TCR signaling and T cell activation [16].

## 6. Effects of vitamin D on CD4+ T cell subsets

Specific T cell subsets (e.g., Th1, Th2, Th17; Fig. 96.1) possess specialized roles in protection against diverse pathogens and immune-mediated disease. Vitamin D influences these subsets both directly and indirectly through effects on dendritic cells, including through modulation of the costimulatory molecules expressed by these cells, and via the soluble mediators they produce.

### 6.1 Th1 cells

Th1 cells play a central role in protection against intracellular pathogens (e.g., viruses, bacteria) where the Th1 signature cytokine, IFNγ, enhances antimicrobial functions of macrophages and other innate cells. Th1 cells are also implicated in human disease and can contribute to immune-mediated pathology caused by uncontrolled inflammation and autoimmune conditions. Vitamin D has consistently been observed to decrease the Th1-associated cytokine, IFNγ, and the Th1-associated transcription factor T-bet [14,17,19]. Recent *in vitro* work suggests that a reduction in IFNγ production, and the concomitant upregulation of the anti-inflammatory cytokine IL-10 can be induced in human CD4+ T cells by both the active and precursor forms of vitamin D, suggesting that these cells can both synthesize and respond to 1,25(OH)<sub>2</sub>D<sub>3</sub> due to their ability to express CYP27B1 [13]. In the experimental system studied, complement signaling, via C3b binding to

its receptor CD46 on CD4+ T cells, drives Th1 differentiation followed by their shutdown. The initial production of IFNγ is followed by expression of IFNγ together with IL-10, and subsequently, IL-10 alone [20]. In follow-up mechanistic studies, CD46 was shown to induce expression of VDR and CYP27B1, leading to the shutdown of the Th1 response, and was proposed as an example of an intracrine pathway in T cells [13], with vitamin D driving this transition through epigenetic modifications and transcription factor (BACH2 and STAT3) recruitment. Further to this, Chauss et al. [13] found that bronchoalveolar lavage (BAL) CD4+ T cells from COVID-19 patients were Th1-skewed, with de-repression of genes that are down-regulated by vitamin D – possibly through vitamin D deficiency or pathway dysfunction. In other studies, analysis of the transcriptomic profile of CD4+ T cells upon interaction with autologous vitamin D<sub>3</sub>-induced tolerogenic dendritic cells showed a significant down-regulation of genes involved in the cell cycle and antigen responses, suggesting the induction of an antigen-specific hyporesponsiveness, in combination with a reduction of the Th1 profile [21]. Conversely, vitamin D has been described to be required for many IFNγ-induced antimicrobial pathways in monocytes and macrophages [22].

### 6.2 Th2 cells

Th2 cells provide protective immunity against helminth infection and facilitate tissue repair, but also contribute to chronic inflammatory diseases such as asthma and allergy, where sensitization increases allergen-specific Th2 and IgE responses [23]. The effects of vitamin D on Th2 responses are complex, with differences reported between studies. The inhibition of Th1 responses by vitamin D in culture is often observed with a reciprocal enhancement of Th2 frequency and increase in the Th2-associated cytokines IL-4, IL-5, and IL-13, as a consequence of the increase in transcription factor GATA3 [17,24]. Some recent studies have recapitulated this finding, including in peripheral blood mononuclear cells (PBMCs) isolated from preeclamptic women [25] and in asthma, where a positive correlation between serum vitamin D status and IL-13 synthesis induced in culture was noted [26]. Similarly in a recent study, human healthy skin explants were exposed *in vitro* to the analog of 1,25(OH)<sub>2</sub>D<sub>3</sub>, calcipotriol, and upon co-culture with allogeneic naïve CD4+ T cells drove differentiation toward a Th2 phenotype synthesizing IL-4 and IL-13 [27]. In contrast, active vitamin D is able to inhibit Th2 cytokine secretion in human cord blood cultures [28].

*In vivo* animal models may help our understanding of these seemingly contradictory observations. Intra-

peritoneal administration of  $1,25(\text{OH})_2\text{D}_3$  had anti-inflammatory effects in an *in vivo* mouse model of asthma, which were attributed in part to the control of the integrin-mediated homing of T cells [29]. In an early life model, *in utero* and early-life vitamin D deficiency introduced via feeding with a vitamin D-low chow exhibited enhanced Th2 responses alongside a reduced T cell IL-10 response in mice, a profile that was enhanced by allergen exposure [30]. The capacity of vitamin D to induce or maintain a range of pathways linked to peripheral tolerance, discussed further in the following, may also be important to our understanding how vitamin D controls Th2 responses. At least one pathway may be specific to the control of Th2 responses, in a study in which vitamin D increased expression of the soluble decoy receptor ST2 by human bronchial and nasal epithelial cells and CD4+ T lymphocytes [15].

Notably most, but not all [26], translational or experimental medicine studies do not show evidence for vitamin D treatment inducing an increase in Th2 cytokine production, or more importantly of exacerbating conditions underpinned by Th2-immunity, such as allergic disease or asthma [5,31]. There is also evidence that supplementation can modulate Th2 responses. For example, Nguyen et al. [32] treated seven cystic fibrosis patients with allergic bronchopulmonary aspergillosis with daily cholecalciferol (4000 IU), albeit without a placebo control group, for 24 weeks and measured production of IL-13 by aspergillus-induced dendritic cell-stimulated CD4 lymphocytes before and after. Decreased IL-13 and IgE responses were observed when compared with baseline.

Importantly, observational studies in children indicate an inverse association between vitamin D status and the development of allergy, including food allergy and asthma [31]. Secondary analyses of two clinical trials (VDAART and COPSAC) of high-dose vitamin D supplementation in pregnancy indicate that achieving vitamin D sufficiency throughout pregnancy reduces the incidence of asthma/recurrent wheeze at 3 years [33,34]. A decrease in allergic sensitizations was also observed in the primary analyses in the VDAART study [35]. In an important longitudinal study that monitored vitamin D status over the first decade of life,  $25(\text{OH})\text{D}_3$  deficiency in early childhood was associated with increased risk for persistent asthma. The authors proposed that plausible contributory factors were modulation of susceptibility to early allergic sensitization, together with effects on upper respiratory tract colonization with bacterial pathogens [36].

### 6.3 Th9 cells

IL-9 is the signature cytokine synthesized by Th9 cells that are associated with anti-helminth and anti-tumor immunity. Regulation of mast cell function, IgE

synthesis, mucus production, and eosinophil maturation by Th9 cells implicates them in immune-mediated and allergic pathology [37]. Vitamin D has been shown to inhibit IL-9 synthesis in an IL-10-dependent manner via a mechanism involving the inhibition of basic leucine zipper transcription factor, a transcription factor important for IL-9 synthesis in Th9 cells [38], and the Th9-associated transcription factor PU.1 [39]. Recent data has also shown that  $1,25(\text{OH})_2\text{D}_3$  is able to attenuate TLR2-mediated differentiation of Th9 cells, a mechanism that appears to involve the downregulation of downstream components of TLR2 including IL-33 and its receptor ST2 [40]. The authors demonstrated that reduced levels of  $25(\text{OH})\text{D}_3$  were inversely associated with enhanced IL9 and TLR2 expression as well as disease severity in patients with rheumatoid arthritis.

### 6.4 Th17 cells

Th17 cells are important for protection against pathogens (e.g., fungi, extracellular bacteria) at mucosal sites, but are also linked to a range of inflammatory conditions, including autoimmune disease and chronic airway illnesses such as severe asthma and COPD.  $1,25(\text{OH})_2\text{D}_3$  is consistently observed to inhibit the production of Th17 cytokines both *in vitro* and *ex vivo* [19,41–46] as well as the expression of CCR6, thus preventing their migration to inflamed tissues. The ability of  $1,25(\text{OH})_2\text{D}_3$  to control Th17 responses may be important in severe steroid-resistant asthma, in which dysregulated Th17 responses predominate [19,26,47]. The effect of vitamin D on Th17 often occurs in parallel to an induction of Treg cells, with  $1,25(\text{OH})_2\text{D}_3$  treatment leading to an upregulation of Treg markers such as the transcription factor, Foxp3, and regulatory cytokines such as IL-10 [14,44,48].

In translational studies, Sotirchos et al. [49] compared individuals with multiple sclerosis receiving daily high-dose (10,400 IU) and low-dose (800 IU) cholecalciferol for 6 months with significantly higher serum  $25(\text{OH})\text{D}_3$  ( $P < .0001$ ) at the end of study for the high-dose group. A decrease in IL-17A + CD4+ T cells and effector memory CD4 + T cells was observed in the high-dose group, with a concomitant rise in central memory and naïve CD4 + T cells when compared with low dose. However, no change in serum cytokine levels was reported.

Drozdenko et al. [46] supplemented individuals with increasing daily doses of cholecalciferol and assessed their lymphocyte phenotype *ex vivo*, compared with control individuals. Supplementation yielded an increase in circulatory CD38 + B lymphocytes and a decrease in IFN $\gamma$  + and IL-17A + CD4 T lymphocyte frequency upon *in vitro* stimulation, with no change in IL-4 and IL-10 producers—possibly altering the balance toward a more Th2 and regulatory phenotype. A similar

modulation of the immune response is also reported by Chambers et al. [26], in a randomized controlled trial in which steroid refractory asthmatics were given oral calcitriol ( $1,25(\text{OH})_2\text{D}_3$ ), or placebo for 4 weeks. Supplementation improved dexamethasone (glucocorticoid) responsiveness *in vitro*, including a decrease in IL-17A and increase in IL-10 production with no change in the inhibition of the IL-13 response. Further to this, IL-10 gene induction within  $\text{CD4}^+$  T cells has been observed *ex vivo*, following calcitriol supplementation, in a study where Urry et al. [50] examined adaptive immune cytokine production from steroid-resistant asthmatic individuals supplemented with calcitriol for several days. Supplementation did not affect lymphocyte gene expression of IL-5, IL-13, or IFN $\gamma$ .

In a study comparing inhibition of T cells responses from the peripheral blood and synovial fluid of patients with active rheumatoid arthritis, Jeffery et al. [51] demonstrated greater inhibition of Th17 cells, with or without IFN $\gamma$  co-expression, by  $1,25(\text{OH})_2\text{D}_3$  from the periphery compared with synovial-derived T cells. The authors suggest that phenotype-committed, inflammatory memory T cells such as those found at inflammatory disease sites may be less susceptible to immunomodulation by vitamin D.

In contrast to the immunomodulatory effects of vitamin D supplementation in children and adults on many effector responses, enhancement has been reported in neonates following vitamin D supplementation (4400 IU/day) during pregnancy [52], although the mechanisms underpinning these differing effects are as yet unknown. *Ex vivo* stimulation of cord blood mononuclear cells (CBMCs) with various innate antigens or the mitogen PHA stimulated a higher production of innate cytokines (GM-CSF, IL-6, IL-8). In T cell stimulation cultures, a fourfold greater IL-17A production and greater dexamethasone-induced IL-10 production was reported [52]. Strong innate immune responses in neonates are associated with a reduced risk of infection and probability of asthma development; these data therefore may indicate that restoring vitamin D sufficiency in pregnancy may improve respiratory health in offspring.

## 6.5 T cell plasticity

The aforementioned studies highlight that plasticity occurs within T cell subsets, which can be regulated by vitamin D. For example, the division of T cells into Th17 and Th1 is not as linear as previously assumed, with data showing that Th17 cells can produce both IL-17A and IFN $\gamma$  in response to IL-12 or TNF $\alpha$  stimulation. These cells express both RORC and T-bet, as well as CCR6 and CXCR3, and have been termed Th17.1 cells

(or nonclassic Th1 cells) [53]. It is hypothesized that these cells contribute to the pathogenesis of autoimmune disease, as they are enriched at inflammatory sites in many diseases [54,55]. These cells can respond to  $1,25(\text{OH})_2\text{D}_3$ ; in CCR6 $^+$  cells,  $1,25(\text{OH})_2\text{D}_3$  reduces the expression of IFN $\gamma$ , IL-17A, and double-positive cells [56]. Similar effects have been observed with Th17.1 cells stimulated in response to air pollution particulate matter [57] and in translational studies in SLE patients supplemented with cholecalciferol [49] putting these observations into a relevant therapeutic context. Another important example of T cell plasticity is the capacity of Th effector cells, such as IFN $\gamma$ -expressing Th1 cells to gain and coexpress antiinflammatory cytokines, typically IL-10, which is influenced by vitamin D [13].

## 6.6 T follicular helper (Tfh) cells

Tfh cells are a  $\text{CD4}^+$  T helper cell population that provide help to B cells to promote the production of high-affinity class-switched antibodies, which are central to effective host protection and underpin vaccine efficacy. Tfh cells regulate B cell function by cell surface molecule interactions (e.g., ICOS, CXCR5, and PD1) as well as by soluble factors. The latter include the signature cytokine IL-21, as well as cytokines linked to other Th subsets including IL-4, historically linked to Th2 cells and known to promote B cell antibody production [58]. The VDR is expressed by Tfh, and was increased, in particular in Th1, Treg, and Tfh cells, in SLE patients in comparison with control subjects [59]. Although we are unaware of reports of how vitamin D acts on Tfh, since  $1,25(\text{OH})_2\text{D}_3$  is known to increase several molecules expressed by Tfh (e.g., Th2 cytokines, ICOS1, PD1), but can inhibit the signature Tfh cell cytokine IL-21 in culture [14], it seems probable that Tfh cell function will also be regulated by vitamin D. A recent study analyzed vitamin D status and immune response in patients hospitalized with moderate and severe COVID-19.  $\text{CD3}^+$   $\text{CD4}^+$  T cells, specifically Th2, Th17, and Tfh, were all reduced in the circulation of patients compared with healthy controls, and vitamin D deficiency was associated with the stimulation of Th2 and the downregulation of Th17 cell but no clear impact on Tfh cells. However, this was only assessed in the periphery, and this reflects the challenges of studying human cell populations that are prominent in tissues [60].

## 7. $\gamma\delta$ T cells

The aforementioned studies report effects of  $\text{CD4}^+$  T cells expressing the  $\alpha\beta$  form of the T cell receptor (TCR). An unconventional T cell subset is defined by

expression of heterodimeric TCRs composed of  $\gamma$  and  $\delta$  chains ( $\gamma\delta$  T cells). They represent a relatively minor T cell subset in peripheral blood, but are enriched in peripheral tissues, such as the skin, intestines, and lungs, and are able to rapidly synthesize large amounts of cytokine and are proposed to play a role in tissue homeostasis and immune surveillance [61]. To our knowledge, little work has been undertaken on the effect of vitamin D in  $\gamma\delta$  T cells. Chen et al. showed that  $\gamma\delta$  T cells express VDR upon activation and that production of IFN $\gamma$  and proliferation were inhibited in response to 1,25(OH) $_2$ D $_3$  [62], and it seems probable that vitamin D will regulate many of these functions both directly and through effects on other cells in their local tissue milieu.

## 8. CD8+ T cells

CD8+ T cells play a role in immune defense against intracellular pathogens (e.g., viruses and bacteria), and for tumor surveillance. They possess cytotoxic activity, which can lead to killing of pathogen-infected and tumor cells. They also secrete significant amounts of cytokines and form comparable subsets, based on cytokine profiles, to CD4+ Th subsets. However, considerably less has been reported about the effects of vitamin D on the CD8+ T cell population than on CD4+ T cells. The impact of vitamin D on cytotoxic or CD8+ T lymphocyte immunity to pathogens and cancer was the focus of a review by Sarkar et al. [63] and is discussed in further detail in [Chapter 95](#).

CD8+ cytotoxic T lymphocytes express VDR and CYP27B1 [12,16] and thus are a target for vitamin D. CD8+ T cells are reported to express relatively high levels of VDR following activation [16,63]. Data from mouse models have suggested that cytotoxic T cells are an important source of CYP27B1, raising the possibility that CD8 T cells may play a role in production of 1,25(OH) $_2$ D $_3$  for paracrine effects [12]. In mice, vitamin D inhibits CD8+ T cell proliferation in culture [64]. Human studies also suggest that vitamin D impacts CD8 T cell expansion and frequency, and this has been studied in the context of human aging. In a comparatively small study of healthy women ( $n = 34$ , all  $> 60$  years of age), increased vitamin D levels were associated with a decline of naïve, but an accumulation of effector, CD8 T cells during early aging [65]. In a much larger study of elderly healthy controls ( $n = 461$ ) and patients with age-related diseases ( $n = 8621$ ), across a range of age-related disease types, one of the most striking associations of vitamin D deficiency ( $< 20$  ng/mL or 50 nmol/L) was with an increased frequency of CD8+ T cells [66].

With regard to T cell phenotypes, in vitro human data have shown 1,25(OH) $_2$ D $_3$  can reduce the frequency of

IFN $\gamma$ -producing CD8 T cells [67] and inhibit skewing toward a Th2 phenotype of IL-13-expressing CD8+ T cells [68], which is at variance with several studies of CD4+ T cells, discussed before. Translational studies have suggested that topical calcipotriol (an analog of 1,25(OH) $_2$ D $_3$ ) treatment of psoriatic lesions could reduce the frequency of CD8+ IL-17+ T cells, resulting in clinical improvement [69]. Vitamin D has also been shown to induce regulatory effects in CD8 T lymphocytes. Recent data suggest that in vitro 1,25(OH) $_2$ D $_3$  treatment, with TGF $\beta$  as a required cofactor, induces the immunoregulatory factor alpha-1 antitrypsin (AAT) production from CD8+ T cells, suggestive of an induction of a CD8 population with anti-inflammatory properties, by vitamin D [70]. Notably, the vitamin D precursor, 25(OH)D $_3$ , was unable to induce a similar effect, suggesting that human CD8+ cells, at least under the experimental conditions used, were unable to generate sufficient active vitamin D.

## 9. Actions of vitamin D on regulatory T lymphocyte responses

Immune homeostasis requires not only the effective induction of immune responses but also their timely resolution. Regulatory T lymphocytes (Tregs) are a vital part of the adaptive immune system and prevent excessive inflammatory responses to pathogens that cause tissue damage and pathology. They promote tolerance to commensal flora and are also crucial in limiting responses to self-antigens and to harmless environmental antigens. A reduction in Treg frequency and/or function has been implicated in the pathogenesis of a number of inflammatory diseases including asthma (reviewed in Ref. [71]) and autoimmune conditions [72]. As discussed in the following, vitamin D positively influences Treg function and frequency and is likely to underpin, at least in part, the beneficial relationship observed between vitamin D and immune-mediated inflammatory disease outcomes. This has generated interest into whether ensuring vitamin D sufficiency through supplementation can reduce the risk of common immune-mediated conditions such as asthma in early life [34,35], and by improving the control of existing disease [5].

Distinct populations of Treg exist. The best described populations express the transcription factor Foxp3 (Foxp3+ Treg) and/or the anti-inflammatory cytokine IL-10. Foxp3+Treg are normally generated in the thymus; however, they can also be induced in the periphery from non-Treg (CD25 negative) CD4+ T cells in the presence of anti-CD3/CD28 antibodies, IL-2, and TGF $\beta$  or retinoic acid. Studies in mice and humans [43,73,74] indicate that vitamin D, specifically 1,25(OH) $_2$ D $_3$  increases the frequency of Foxp3+



expressing T cells with inhibitory function. This may be through effects on dendritic cells [75–77], as well as direct actions on T cells [43,74]. Kang et al. [78] reported that  $1,25(\text{OH})_2\text{D}_3$  promotes FOXP3 expression via binding to vitamin D response elements in its conserved non-coding sequence region.

IL-10 expressing Treg cells with suppressive function are also induced by vitamin D in culture, potentially directly and indirectly, via actions on dendritic cells [79]. In more recent reports,  $1,25(\text{OH})_2\text{D}_3$ , alone or together with TGF $\beta$ , induced expression of the serine protease inhibitor,  $\alpha$ -1 antitrypsin by human CD4 [80] and CD8 T cells [70].  $\alpha$ -1 antitrypsin has a range of immunomodulatory properties and IL-10 induction by  $1,25(\text{OH})_2\text{D}_3$  in CD4+ T cells appears partially dependent on the  $1,25(\text{OH})_2\text{D}_3$ -mediated increase in  $\alpha$ -1-antitrypsin [80]. In addition, effector T cells exhibit plasticity and can be induced to co-express anti-inflammatory IL-10 alongside effector cytokines (e.g., IL-17A, IFN $\gamma$ ), which are likely to contribute to the resolution of immune responses [13]. In this system, vitamin D acted on CD4+ T cells activated via the complement system, to drive the transition from single IFN $\gamma$  positive T cells to cells co-expressing IFN $\gamma$  with IL-10 and then single IL-10+ cells. In an elegant and in depth study, the authors demonstrated that vitamin D caused genome-wide epigenetic remodeling and induced and recruited transcription factors (e.g., STAT3, c-JUN, BACH2). These were shown to repress Th1 and Th17 programs and induced IL-10 via IL-6-STAT3 signaling [13].

Many of these original reports of the effect of vitamin D on human Treg arise from laboratory studies and are supported by robust data in animal models. Importantly, translational evidence exists supporting many of these effects in vivo in humans. For example, in asthma, circulating vitamin D levels have been shown to positively correlate with the IL-10 levels and frequency of Foxp3+T cells in pediatric bronchoalveolar lavage [74,81], as well as Foxp3+Treg number in the periphery in adult patients with moderate to severe asthma [82].

Tregs inhibit the function of other lymphocytes, APCs, and innate and structural cells to dampen immune responses through a variety of inhibitory mechanisms. These range from the synthesis of inhibitory cytokines such as IL-10 and TGF $\beta$ , through to metabolic disruption and competition for essential growth factors such as IL-2, by granzyme/perforin-mediated cell cytotoxicity, to effects on the maturation and function of APCs (reviewed in Refs. [72,83]).  $1,25(\text{OH})_2\text{D}_3$  upregulates many of these functions, including inhibitory molecules that Treg are known to express such as CTLA4, PD-1, CD73, and CD200, an area that has been extensively reviewed [3,73].

Our understanding of the breadth of Treg function is expanding, and this brings new perspectives of how vitamin D may influence Treg function and human health. Specialized Treg are reported to be widely present in tissues including visceral adipose tissue, colon, skin, lung, skeletal muscle, and placenta [84–86] where they promote wound healing and tissue repair. For example, in the skin, which is an important site of vitamin D production and metabolism and where Tregs are abundant, Treg may facilitate not only wound healing and repair but also hair morphogenesis and tolerance to skin commensals. Obesity is associated with low serum levels of  $25(\text{OH})\text{D}_3$ , and in adipose tissue, resident Tregs are proposed to regulate adipose tissue homeostasis and limit obesity-associated systemic inflammation [87]. The impact of vitamin D in these tissues, and specifically how  $1,25(\text{OH})_2\text{D}_3$ -mediated effects on adaptive immunity and Treg regulate tissue homeostasis more widely, is an important and evolving area of interest.

Translational studies continue to demonstrate associations between Treg parameters and  $25(\text{OH})\text{D}_3$  status in humans, beyond those described in asthma above, and suggest that maintaining vitamin D sufficiency has the potential to incrementally improve disease outcomes in a range of immune-mediated inflammatory conditions. New approaches are emerging such as a recent study of the use of an injectable hydrogel platform for sustained delivery of anti-inflammatory nanocarriers, and the induction of Treg in atherosclerosis in mice. In this system, the impact of a single slow release bolus of  $1,25(\text{OH})_2\text{D}_3$  may provide advantages to more frequent delivery of soluble  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$  [88]. The immunoregulatory and anti-inflammatory properties of vitamin D continue to be explored as an adjuvant in antigen-specific desensitization therapies [89].

In conclusion, translational studies indicate that vitamin D acts upon T lymphocytes and diminishes IL-17 and IFN $\gamma$ , while enhancing IL-10 responses and Foxp3 Treg frequency not only in the periphery but also in tissues such as the airways. Long-term vitamin D supplementation in multiple sclerosis patients has also been reported to enhance serum TGF $\beta$ -1, although it is unclear whether this derives from lymphocytes or other sources [90]. There is, however, an absence of robust evidence that vitamin D therapies enhance Th2 responses in humans in vivo, although a decrease in Th1/17 frequency without alterations in Th2 inflammation might shift the immune equilibrium in the direction of the latter. In addition, many immunoregulatory functions are reported to be upregulated by  $1,25(\text{OH})_2\text{D}_3$ . These have been described in experimental cultures and frequently supported by in vivo correlates between vitamin D status and specific immune parameters and

following vitamin D treatment of patients. These data argue, plausibly, that despite *in vitro* induction of Th2 responses by vitamin D, the failure to support these data *in vivo* within humans and link them to worsening of Th2-mediated conditions [31] may be because this is balanced against the enhancement of relevant immunoregulatory functions.

## 10. Actions of vitamin D on B lymphocytes

B lymphocytes are key players of the adaptive immune system; they elicit T lymphocyte help and produce pro- and anti-inflammatory cytokines. B cells are characterized by their synthesis of antibodies or immunoglobulins (IgM, IgD, IgG1-4, IgA, and IgE), which are integral to the humoral immune response. Immunoglobulins are central for defense against extracellular pathogens and the efficacy of essentially all vaccine-induced protection to pathogens. However, B cells and antibodies also play a prominent role in allergic and autoimmune disease.

Similar to T lymphocytes, B cells upregulate expression of both VDR and CYP27B1 following activation and are able to synthesize  $1,25(\text{OH})_2\text{D}_3$  [91,92]. However, there are discrepancies between the effect of  $1,25(\text{OH})_2\text{D}_3$  on B cell function *in vitro* and in patient association studies. For example,  $1,25(\text{OH})_2\text{D}_3$  inhibits proliferation and induces apoptosis *in vitro*, while also reducing total IgM and IgG and plasma cell frequency [91,93–96]. These observations have not been recapitulated *in vivo*, in patients with autoimmune disease, as reviewed by Rolf et al. [95].

Of interest in the context of allergic and asthmatic disease, vitamin D treatment of peripheral human B cells in culture reduces IgE production, inhibits antigen presentation functions, and switches toward a regulatory phenotype through increased IL-10 production [92,97,98]. Active vitamin D is observed to inhibit IgE production *in vitro* [96], as well as inhibiting proliferation and differentiation into IgE-, IgG-, and IgA-producing plasma cells and reducing memory B cell formation [97]. The inhibition of IgE class-switch recombination in human B cells is through VDR recruitment of a transrepressive complex to the  $\epsilon$ -germline gene promoter [96].

Many studies on the effect of vitamin D on IgE have been undertaken in mice, with VDR or CYP27B1 knockout mice exhibiting elevated IgE, leading to the conclusion that endogenous calcitriol plays a role in the regulation of IgE, with the potential to limit the development of detrimental allergic responses [99,100]. Observational studies have assessed correlates between IgE and vitamin D status. Hypponen et al. in a large study cohort (> 7000) reported a nonlinear relationship,

with both extremely high, albeit with very low numbers of individuals, and low levels of  $25(\text{OH})\text{D}_3$  associated with elevated IgE concentrations [101]. Gupta et al. [102] reported, in a pediatric cohort, a significant inverse relationship between vitamin D status ( $25(\text{OH})\text{D}_3$ ) with total serum IgE, IgE with specificity for a panel of 5 different aeroallergens, as well as the sum of specific IgE to aeroallergen, but not to sum of specific IgE to food allergens. In a larger cohort, in the National Health and Nutrition Examination Survey 2005–06, vitamin D deficiency was associated with higher levels of IgE sensitization in children and adolescents, including to the food allergen peanut. Notably, no consistent associations were seen between  $25(\text{OH})\text{D}_3$  levels and allergic sensitization in adults [103]. However, vitamin D supplementation (4000 IU/day), compared with placebo for 4 weeks, had no significant effect on serum levels of total IgE, IgE to dust mite, or IgE to cockroach in children with asthma and low vitamin D levels with mean duration of follow-up of 316 days [104].

IgA is required for mucosal immunity, and calcitriol has been observed to control its production in purified human B cells. Notably, calcitriol together with 9-*cis* retinoic acid induced the differentiation of naïve B cells into plasmablasts secreting IgA. This is suggested to require recruitment of the VDR to the TGF- $\beta$  promoter [105]. These two nuclear hormone receptor ligands were proposed to act in an additive, rather than a competitive signaling manner. The authors propose these data suggest that the VDR is a major driver of B cell activation and differentiation, with a potential to channel toward a more protective response.

## 11. Actions of vitamin D on the immune microenvironment and the function of APCs

The capacity of vitamin D to modulate the function of APCs and other cell types, including dendritic cells and macrophages, and structural cells within the local tissue environment, will in turn influence lymphocyte responses. Dendritic cells likely represent an important source of  $1,25(\text{OH})_2\text{D}_3$ , through their expression of CYP27B1 [106,107]. The consequences of vitamin D actions on dendritic cells can result in the failure to activate T cells as robustly, leading to reduced effector responses, and/or to induce tolerance and regulatory T cells.

$1,25(\text{OH})_2\text{D}_3$  has well-documented effects on dendritic cells that influence subsequent immune responses. It acts to maintain an immature dendritic cell phenotype and expression of the monocyte marker CD14, together with reduced expression of CD80/86 and HLA-DR—proposed to be through the inhibition of NF- $\kappa$ B p65 phosphorylation in myeloid, but not plasmacytoid dendritic cells [108,109]. Vitamin D inhibits cytokines

synthesized by dendritic cells that are known to influence T cell phenotype, including two members of the IL-12 family, specifically, IL-12 that drives Th1 responses, and IL-23, which is involved in the maintenance and expansion of Th17 cells [57,110]. This is of relevance in human diseases since Th17 cells in the presence of these cytokines often play a pathogenic role [111].  $1,25(\text{OH})_2\text{D}_3$  also upregulates expression of inhibitory costimulatory molecules, PD-L1 [79] and ILT3 [76], as well as reduced secretion of CCL12 and CCL22, resulting in reduced chemotaxis [109]. Recent data suggest that the mechanism of vitamin D actions involves reprogramming of dendritic cell glucose metabolism [112], fatty acid synthesis [113], and activation of the IL-6-JAK-STAT3 pathway [114]. Of note, pretreatment with vitamin D appears to be required for optimal suppression of proinflammatory responses to TLR stimulation [115]. These data suggest that improving long-term vitamin D status will be required to modulate immune phenotype, and extended periods of supplementation may be required.

Such tolerogenic (immature) phenotypes of dendritic cells induce IL-10-secreting Treg differentiation, elevated IL-10 production, reduced inflammatory cytokines, and reduced proliferation [109,116]. Tolerogenic dendritic cells, induced by a short treatment with VDR agonists, promote  $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$  Treg cells that are able to mediate transplantation tolerance and to arrest the development of autoimmune diseases [75]. More recently, dendritic cells engineered to produce high concentrations of  $1,25(\text{OH})_2\text{D}_3$  and vitamin A (retinoic acid) augmented the induction of  $\text{Foxp3}^+$  T cells that express the gut-homing receptor CCR9 in vitro as well as in vivo, where they homed to the intestines and could suppress experimental colitis [77]. Some of the proposed mechanisms by which vitamin D may promote tolerance through dendritic cell modulation include expression of PD-L1, IL-10 production, increased expression of membrane bound TNF, and the control of proliferation of inflammatory cell types [79,112,117]. Despite these advances, the mechanism by which vitamin D—treated tolerogenic dendritic cells are able to induce Treg cells remains incompletely understood.

In addition to the effects on antigen presentation, vitamin D can control the levels of soluble mediators produced by other cells in the immune microenvironment including IL-10, IL-12, and IL-23 (discussed before), thereby governing lymphocyte responses, as well as chemokines governing lymphocyte homing [107,118].

Dendritic cells, bronchial epithelial cells, and keratinocytes can also produce  $1,25(\text{OH})_2\text{D}_3$  through their expression of CYP27B1, while  $1,25(\text{OH})_2\text{D}_3$  produced by monocyte-derived dendritic cells has been shown to enhance CTLA-4 expression and reduce IL-17,  $\text{IFN}\gamma$ , and IL-21 expression in cocultured T lymphocytes [14].

Furthermore, both IL-6 and  $\text{TGF}\beta$  can be regulated by vitamin D, both of which modulate regulatory T cell responses [119–121]. Finally, vitamin D can regulate the IL-33 pathway. IL-33 is a proinflammatory mediator released by damaged epithelial cells, which can promote Th2 responses. The soluble receptor (sST2) can antagonize the actions of IL-33. Both  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$  have been observed to stimulate sST2 production by human bronchial and nasal epithelial cells, while  $\text{CD4}^+$  T lymphocytes produce sST2 in response to  $1,25(\text{OH})_2\text{D}_3$ , but not  $25(\text{OH})\text{D}_3$  (reflective of their low CYP27B1 expression)—thereby inhibiting the actions of IL-33 in culture [15]. This might be predicted to be beneficial in allergic disease but could be counter-indicated in other conditions.

## 12. Effects of vitamin D upon immunometabolism in APC and T cells

One proposed mechanism by which vitamin D alters dendritic cell and T cell phenotype and function is through the modulation of immunometabolism. In recent reports, the immunomodulatory effects of vitamin D have been attributed to its capacity to regulate both fatty acid and glucose metabolism by altering glycolysis, tricarboxylic acid (TCA), and oxidative phosphorylation (OxPhos) rates [108,112,113,122]. Antigen stimulation within immune cells is established to trigger high rates of aerobic glycolysis to support clonal expansion and effector cytokine production [123]. This state of high aerobic glycolysis, known as the Warburg effect, is an adaptation frequently observed in cancer—a disease model in which vitamin D has been found to reduce aerobic glycolysis [124]. On the other hand, vitamin D given during dendritic cell differentiation into tolerogenic dendritic cells has also been found to be dependent upon increases in glycolysis and fatty acid synthesis, albeit with concomitant rises in OxPhos [108,112,113,122].

A decrease in glycolysis has been reported in  $\text{CD4}^+$  effector T cells upon vitamin D treatment, with associated changes in effector function ( $\text{IFN}\gamma$  production), with a lesser response upon oxidative phosphorylation than that reported in dendritic cells [125]. The metabolic effects of vitamin D upon T cells were highlighted through associations with low serum vitamin D and high metabolic rates in PBMCs, followed by findings that  $1,25(\text{OH})_2\text{D}_3$  treatment regulates metabolic regulators, such as c-Myc and mTOR pathway constituents [126–128]. This potential of calcitriol to modulate T cell metabolism may aid in the polarization toward Treg, over a more inflammatory Th17 phenotype [129]. From these data, vitamin D has evident effects upon immunometabolism and signaling pathways such as PI3 kinase/

AKT/mTOR. However, this is a complex field, and reports indicate that responses may differ between cell type, differentiation status, and other variables. Further studies are awaited with interest.

### 13. Translational aspects of vitamin D regulation of adaptive immunity—vitamin D and vaccine responses

Since their introduction, vaccinations have saved and improved the quality of countless lives; however, subgroups of the population exist for whom immune responses to vaccination remain poor, including the very young, elderly, and immunocompromised individuals. This has been further highlighted following the global rollout of COVID-19 vaccinations, to which immunosuppressed individuals exhibit low seroconversion [130], while a recent report suggests that vitamin D sufficiency is beneficial for seroconversion to the SARS-CoV-2 BNT162b2 vaccine [131]. Vaccine efficacy is predominantly reliant on robust, high-affinity antibody responses, which neutralize viruses or toxins, and opsonize pathogenic bacteria. Serum IgG and in some cases additionally IgA (e.g., rotavirus vaccine responses) at mucosal sites are crucial for vaccine success. Most vaccine-induced antibody responses require the help of CD4<sup>+</sup> T cells, particularly Tfh cells—the exception to this being the pure polysaccharide vaccines, which are so-called T cell independent.

The mechanism by which vitamin D may regulate responses to vaccination is plausibly through modulation of both the innate and adaptive immune response. Rapid antigen detection at the vaccination site by dendritic cells and other APCs, migration to draining lymph nodes, and presentation to antigen-specific lymphocytes are required for efficient immunization, a mechanism that paracrine production of calcitriol following TLR ligation was shown to improve in adult mice [132]. One might predict the tolerogenic effect of vitamin D upon APCs and T cells, discussed before, to have a negative impact upon response to vaccination. However, for optimal vaccine responses, class switching from IgM- to IgG-producing B cells is required, and IL-10 secretion has been shown to induce this [133]. Tfh cells are also required for optimal B cell responses to vaccines at the germinal center; however, extensive literature regarding the effects of vitamin D upon these cells is still lacking. There remains limited research into the outcomes of vitamin D treatment upon B cell responses to vaccination [92,97].

Seasonality and latitude are often used as a surrogate for vitamin D status. For example, response to summer inoculation with rubella vaccine in children is modulated when compared with winter, suggesting that higher vitamin D may reduce immunogenicity [134]. However, the immunological effects of ultraviolet B (UVB) generated vitamin D versus supplementation

are not entirely comparable (reviewed in Refs. [135,136]). In contrast, vitamin D deficiency is associated with poor response to measles, hepatitis B, and BCG vaccinations, implying that vitamin D status and UV exposure may not directly align, or that the immunological effects of vitamin D may vary depending upon antigen stimulus [135,137–140]. In the context of influenza vaccines, a systematic review and metaanalysis of studies by Lee et al. [141], to investigate a role for vitamin D<sub>3</sub> deficiency in seroconversion and seroprotection rates, found no significant associations between vitamin D<sub>3</sub> status and vaccine response. They did, however, find that deficient individuals had lower seroprotection rates than sufficient individuals for some strains.

Supplementation studies are fundamental to understanding how vitamin D status may alter or improve vaccination effectiveness, some of which have been reviewed elsewhere [142]. More recently, Goncalves-Mendes et al. [143] investigated the effects of supplementation (100,000 IU/15 days, for 3 months) in elderly (> 65 years of age) vitamin D-deficient individuals upon response to influenza vaccination using a randomized placebo-controlled double-blind trial. Restoring sufficiency did not increase antibody titer, although it did increase Th2/Th1 balance in favor of the former.

### 14. Disparity between association, translational, and clinical studies

Epidemiological studies linking vitamin D status with various health outcomes are frequently indicative of a beneficial effect. Experimental medicine studies of the correlation of vitamin D status or the impact of vitamin D supplementation largely support experimental findings on the impact of vitamin D on adaptive immune parameters, as discussed before, and in many cases plausibly explain epidemiological observations. For example, in chronic airway conditions such as asthma, vitamin D sufficiency is linked to reduced risk for the development of asthma in early life [31,33,36], reduced frequency of exacerbations, frequently caused by respiratory viral infections, and improved responsiveness to treatments such as steroids [5]. Immunologically, 1,25(OH)<sub>2</sub>D<sub>3</sub> is known to enhance a range of antimicrobial pathways (see Chapters 94 and 95). Likewise, as detailed earlier in this chapter, 1,25(OH)<sub>2</sub>D<sub>3</sub> can dampen inflammation, promote pathways associated with peripheral tolerance, and improve the clinical and immune (e.g., reduce Th17 response linked to severe, treatment refractory asthma and boost IL-10 and Foxp3 Treg) response to steroids [5,26,50]. Despite this, clinical findings from vitamin D supplementation trials are frequently inconclusive. Examples of this are given in Table 96.1, in which the details and outcomes of various clinical trials pertaining to the effects of vitamin D supplementation in asthma are shown.



**TABLE 96.1** Study outcomes of vitamin D supplementation in asthma.

Participants	Intervention	Outcomes	References
48 newly diagnosed asthmatic children	500 IU/day cholecalciferol or placebo for 6 months	Significant decrease in exacerbations in vitamin D group	[144]
20 children with chronic asthma	1000 IU/day cholecalciferol or placebo for 1 year	No significant difference in lung function (FEV1) or asthma control test score	[145]
100 moderate–severe asthmatic children	60,000 IU cholecalciferol per month or placebo for 6 months	Significantly reduced exacerbations and corticosteroid requirement in vitamin D group	[146]
130 asthmatics	100,000 IU bolus cholecalciferol intramuscularly plus 50,000 IU/week for 24 weeks or placebo	Significantly improved lung function in the vitamin D group compared with placebo	[147]
23 severe steroid-refractory adult asthmatics	0.25 µg/mL calcitriol twice daily or placebo for 4 weeks	Significantly improved lung function response to prednisolone in vitamin D group	
408 adult asthmatics (VIDA)	100,000 IU single-dose cholecalciferol followed by 4000 IU/day or placebo for 28 weeks	No significant difference in time to first asthma treatment failure; significantly lower inhaled corticosteroid requirement at study end in vitamin D group; borderline significant reduction in cumulative exacerbations with vitamin D supplementation	[148]
250 adult asthmatics (VIDA)	120,000 IU cholecalciferol or placebo every 2 months for 12 months	No significant difference in time to first asthma exacerbation	[149]
44 nonatopic sputum neutrophilic and/or eosinophilic asthmatics	Single-dose 400,000 IU cholecalciferol or placebo. Assessed 9 weeks later	No significant overall difference in sputum neutrophil or eosinophil counts Significantly reduced sputum eosinophils in supplementation group with highest baseline eosinophil levels	[150]
89 asthmatic children	800 IU/day cholecalciferol or placebo for 2 months	Significantly improved asthma control in vitamin D–treated group	[151]

**TABLE 96.1** Study outcomes of vitamin D supplementation in asthma.—cont'd

Participants	Intervention	Outcomes	References
<b>231 moderate–severe asthmatic children</b>	300,000 IU (<5 years) or 600,000 IU (>5 years) intramuscular calciferol, then 400 IU/day cholecalciferol, versus with 400 IU/day only group	No significant decrease in exacerbations over 12 months	[152]
<b>192 moderate–severe asthmatic children</b>	4000 IU/day versus placebo, alongside inhaled fluticasone for 12 months	No significant decrease in exacerbations over 12 months	[153]
<b>250 persistent asthmatic children</b>	1000 IU/day or placebo for 9 months	No significant change in childhood asthma control test score	[154]
<b>112 adult asthmatics</b>	16,000 IU/week calcifediol versus placebo, for 6 months	Vitamin D supplementation significantly improved asthma control test scores	[155]

Divergences between epidemiological and experimental data with clinical outcomes are likely to be due to a combination of factors. Broadly differing participant groups, dosing regimens, including the form of vitamin D prescribed, supplementation methodologies and study endpoints, as alluded to above, are all likely to contribute to this lack of concordance between epidemiology and translational studies. Other possibilities are that elevated serum 25(OH)D<sub>3</sub> levels could be reflective of increased UV exposure and health benefits could be due to vitamin D–independent and UV-dependent mechanisms. Supplementation doses (and/or compliance) often fluctuate greatly, and this could lead to insufficient, or poorly sustained, systemic vitamin D levels, and therefore, shorter dosing intervals with a higher vitamin D dose may be required [156]. Employing different forms of vitamin D could cause more variation in clinical outcomes, as vitamin D intake and serum levels are not always linear [157]. This includes the use of vitamin D<sub>2</sub> versus vitamin D<sub>3</sub>, as their capacity to alter serum 25(OH)D<sub>3</sub> levels and effects upon the immune system are not identical [9,158]. Durrant et al. [9] reported that blood transcriptome analysis following D<sub>2</sub> or D<sub>3</sub> supplementation differentially altered gene expression, with D<sub>3</sub> downregulating the majority of genes, while upregulating type I and II interferon-related genes—a response not observed with D<sub>2</sub> supplementation. Ethnicity can also have an impact upon serum vitamin D levels. Proposed mechanisms for this are decreased UVB absorption in those with darker skin pigmentation, differences in VDR pathway gene expression/polymorphisms, or differences in

parathyroid hormone (PTH) that stimulates renal CYP24A1 expression [9,159,160]. 25(OH)D<sub>3</sub> can, in fact, inversely correlate with 1,25(OH)<sub>2</sub>D<sub>3</sub>, and polymorphisms within vitamin D pathway genes or disease risk genes that may facilitate beneficial immune effects of vitamin D (i.e., 17q21 locus in asthmatic individuals) may confound clinical outcomes [161,162]. Circulating serum vitamin D levels can also decrease during inflammatory responses, as vitamin D is converted to active calcitriol locally [8]. This could indicate that higher levels of vitamin D are required for individuals with inflammatory conditions, such as those highlighted in epidemiological studies.

Conditions that are a result of immune dysregulation, such as asthma, for which vitamin D has been highlighted as a potentially beneficial therapy are often heterogenous in their presentation. To this end, various biologic therapies have failed to be successfully transferred from proof-of-concept studies to clinical trials, unless in well-defined patient endotypes [163]. This may also apply in vitamin D trials, for example, targeting specifically those individuals with significant deficiency at baseline may demonstrate better outcomes—as has been implied by findings in chronic obstructive pulmonary disease [164,165]. It is therefore conceivable that the absence of beneficial vitamin D effects within clinical trial data could be due to inadequate trial design, namely dosing schedules and patient stratification. It is also essential to recognize that diseases that have a component of immune dysregulation cannot be simply attributed to disruption of the adaptive immune system. Applying the pathophysiology of asthma

as an example, in addition to adaptive immune dysregulation, bronchial smooth muscle undergoes hypertrophy, airway remodeling occurs, while the respiratory epithelial integrity becomes compromised. How vitamin D supplementation affects these facets of disease needs to be considered in trial designs.

Restoration of vitamin D sufficiency may not, or only modestly, improve disease symptoms but could alter the local inflammatory milieu to facilitate improved responses to other therapies, as indicated for asthmatic treatment with inhaled corticosteroid treatment, in antimicrobial treatment of tuberculosis and in allergen immunotherapy [50,166,167]. Finally, the age at which vitamin D status restoration occurs is an area where further clinical data is required as, while vitamin D appears to have immunomodulatory effects upon adaptive immunity, various studies investigating the effects of vitamin D supplementation in pregnancy suggest that sufficiency can bolster the immune response in the neonate and early life [35,168].

### 15. Protection from exacerbation of airway disease by vitamin D

While the protective effects of vitamin D are explored to a greater extent in Chapter 44 “Vitamin D and the lungs,” here we will discuss a role for vitamin D within the adaptive immune component of airway disease exacerbations. Exacerbations of chronic airway respiratory disease are commonly caused by respiratory viral infections, and protective effects of vitamin D are likely to be linked to its capacity to enhance antimicrobial pathways [5]. However, periods of poor air quality are associated with increased lower respiratory infections and increased respiratory exacerbations within individuals with chronic respiratory conditions [169–171]. Several studies have associated increased air pollutant concentrations with vitamin D deficiency [172–174]. These pollutants included fine and coarse particulate matter (PM<sub>2.5</sub> and PM<sub>10</sub>, respectively) and nitrogen oxides (NO<sub>x</sub>). Further to this, Bose et al. [175] associated low vitamin D status with increased respiratory episodes in obese asthmatic children in areas with high PM<sub>2.5</sub> concentrations, while a higher vitamin D status was protective against asthmatic symptoms. These associations are frequently attributed to the attenuation of UVB intensity at ground level by high pollutant concentrations, though other inflammatory factors may contribute.

Individuals with chronic respiratory conditions also have hampered natural antioxidant defenses, leaving them particularly susceptible to PM-induced respiratory epithelial damage and inflammation [176]. Oxidative stress is a well-established pathophysiological mechanism of air pollution, and this, even in the absence of

PM, has been found to alter both dendritic cell and lymphocyte responses [177]. For example, increases in oxidative stress (decreased glutathione levels) upregulate costimulatory molecules and IL-12 within dendritic cells and subsequent CD4 T cell expansion in response to LPS, as well as increased IFN $\gamma$  expression in mice with heightened oxidative stress, indicating that an oxidative environment may favor Th1 inflammation [178,179]. At the respiratory epithelium, PM decreases antioxidant defenses and stimulates innate and adaptive immune cell infiltrate, as observed in various in vitro and in vivo human exposure studies [180–182]. Pfeffer and colleagues [182] found that vitamin D pretreatment was protective against PM-stimulated human bronchial epithelial cell (HBEC) inflammation, while bolstering antioxidant defenses and decreasing GM-CSF release by healthy HBECs.

PM pollution can directly alter the phenotype of APCs in vitro and in vivo, inducing dendritic cell maturation, which indirectly impacts CD4 and CD8 lymphocyte responses to exacerbate airway inflammation [57,182,183]. As discussed before, vitamin D promotes a tolerogenic dendritic cell phenotype. Confirming this in the context of air pollution, Mann et al. [57] reported that reference urban PM activated dendritic cells by increasing costimulatory molecule and MHC II expression, which, in turn, drove an IL-17A + IFN $\gamma$  + response in coculture with autologous memory CD4 T cells. This was offset by pretreatment with 1,25(OH)<sub>2</sub>D<sub>3</sub>, in an IL-23-dependent mechanism. In conjunction with epidemiological studies, these mechanistic findings position vitamin D as a promising, readily available, and low-cost therapeutic to counter, at least partially, the adverse effects of air pollutants upon adaptive immunity.

### 16. UVB and vitamin D at the skin—how does this compare with oral supplementation

Vitamin D is largely produced following UVB irradiation of the skin via photolysis of 7-dehydrocholesterol (7-DHC), and its production can therefore be affected by the time/intensity of exposure, clothing, skin pigmentation, and polymorphisms in various enzymes involved in cholesterol synthesis [160]. It is unclear how immunoregulatory properties of vitamin D generated by UVB irradiation differ from vitamin D supplementation (reviewed in Refs. [135,136]); however, it is unlikely that epidemiological studies alone will resolve this question. For example, one argument is that epidemiological associations between low vitamin D status and immunological conditions could be as a result of reverse causation. Reverse causation suggests that systemic inflammation can cause decreases in circulating

vitamin D levels, but also that individuals with immunological conditions may undergo less sunlight exposure [184]. There is, however, limited data available to support this latter theory, while increasing outdoor sunlight exposure can be inadequate in obtaining vitamin D sufficiency in urban children with asthma [175]. Whether vitamin D obtained through supplementation has comparable beneficial effects to those of UVB irradiation-generated vitamin D remains unclear. UV radiation can generate other mediators that may also cause immunomodulation, rendering it difficult to determine the vitamin D–dependent outcomes, and this has been explored in animal models (reviewed in Ref. [135]). Mechanistic studies investigating the effects of UVB irradiation in vivo are limited, particularly within asthmatic individuals, although Malerba et al. have reported in an asthmatic patient suffering from psoriasis that IL-17A and Th1 cytokine expression decreased following UVB phototherapy [185]. This indicates that irradiation can generate similar immunomodulation to that, which is frequently reported from in vitro and translational studies. UV radiation has also been reported to be beneficial in multiple sclerosis, despite using vitamin D status and latitude as a measure of UV radiation levels, thus not considering how vitamin D levels decline with inflammation [186]. Ostkamp et al. [186] also carried out transcriptome analysis upon immune cells from narrowband irradiated patients, in which vitamin D and type I IFN signatures were increased in monocytes, B and T cells, although it is not known if these two signatures have a causative relationship.

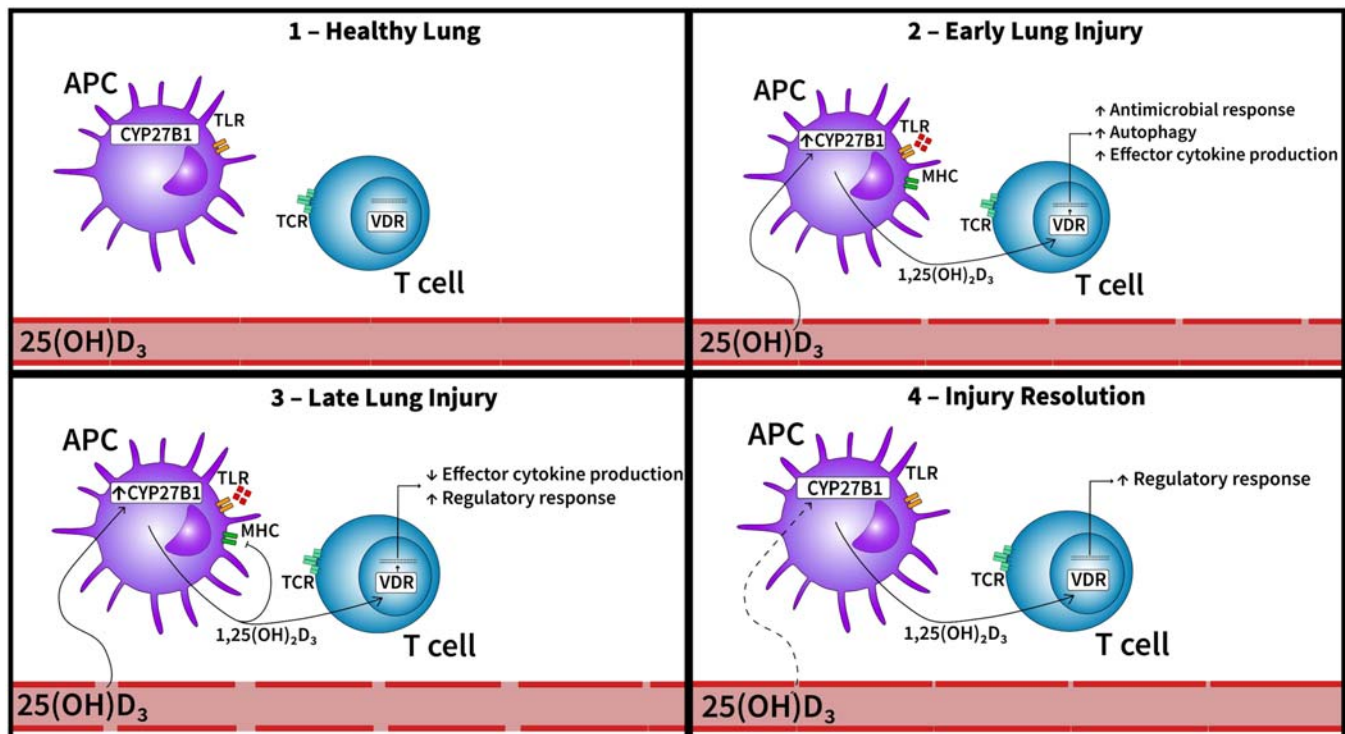
Immune cells at the skin represent a unique population that likely have a higher exposure to higher concentrations of vitamin D than immune cells elsewhere within the body. It is, however, worth noting that vitamin D needs to be converted to 25(OH)D<sub>3</sub> before it can be converted to the active calcitriol—whilst the majority of this occurs within the liver by CYP2R1, other cells at tissue sites, including skin dendritic cells, are capable of this and likely to impact local adaptive immune responses [107]. Langerhans cells (LCs) are tissue-resident dendritic cells, which are a target of UV exposure. Murine models have shown that UV irradiation results in LC migration to draining LNs and subsequent Treg induction [187,188]. UV irradiation in mice has also been found to cause immunomodulation of bone marrow–derived dendritic cells, indicative that UV irradiation can have systemic effects—possibly by elevating vitamin D concentrations [189]. However, it is unclear if these responses are truly related to changes in vitamin D concentration as mice lacking the VDR gene have similar levels of Treg induction following UV irradiation to those with wild-type VDR expression [190]. Establishing whether findings from animal models are translatable into mechanisms of human

disease is helpful, and psoriasis is one condition in which UV/vitamin D research is concentrated. Psoriasis is an autoinflammatory disease of the skin, within which Th1/17 inflammation is strongly implicated and is one of the few conditions in which UV radiation is well established to have beneficial and immunomodulatory effects in humans [191–193]. In addition to this, topical VDR agonists (calcipotriol) are used as effective therapeutics, though more evidence is required to confirm that both topical VDR agonists and irradiation have the same effects as increasing systemic vitamin D levels through supplementation [194]. McCullough et al. [195] compiled results from various studies investigating the effects of either oral or topical vitamin D, or sunshine and UVB phototherapy in psoriasis to reveal comparable beneficial outcomes, including in patients with “normal” baseline serum 25(OH)D<sub>3</sub> levels. Overall, these data confirm a beneficial role for oral, topical, and UV-derived vitamin D, although there are likely vitamin D–independent benefits of UV exposure too.

## 17. Conclusions

In conclusion, it is evident that vitamin D has modulatory properties in adaptive immunity; however, VDR expression and subsequent signaling is also required for efficient initiation of classical T cell receptor signaling within naïve T cells [16] and likely other cell types also [105]. This, in addition to other reported functions such as antimicrobial properties, and bolstering of immunity in the neonate positions vitamin D as a unique facilitator of both immune activation and suppression [196]. We therefore envisage that vitamin D acts as a spatial–temporal regulator of adaptive immune responses, upon infection and tissue injury to help initiate efficient, but not excessive, immune responses—see Fig. 96.2. As tissue injury or infection occurs, innate mediators are released that in turn increase vascular permeability, generating a local pool of 25(OH)D<sub>3</sub>. In addition to this, upon exposure to pathogen-associated molecular patterns, Toll-like receptor (TLR) ligation triggers the upregulation of CYP27B1 to accelerate conversion of 25(OH)D<sub>3</sub> into active 1,25(OH)<sub>2</sub>D<sub>3</sub> [197]. The increased levels of active vitamin D would facilitate enhanced lymphocyte activation, antimicrobial peptide production, and autophagy [198] to aid pathogen clearance [112,196]. Finally, as inflammation progresses, a transition to a proresolving environment would occur, increasing IL-10 expression and inhibiting Th1/Th17 inflammation, while the expression of CYP24A1 would form a negative feedback loop to catabolize local active vitamin D. In the absence of sufficient 25(OH)D<sub>3</sub>, such as in vitamin D–insufficient and vitamin D–deficient individuals, inflammation may be sustained and a





**FIGURE 96.2** Vitamin D as a spatiotemporal regulator of adaptive immune responses at sites of inflammation, such as the lung epithelium. We hypothesize that in the healthy airways of vitamin D-sufficient individuals (1), there are low levels of available  $25(\text{OH})\text{D}_3$  and PAMPs, thereby maintaining a baseline expression of TLRs and 1- $\alpha$ -hydroxylase (CYP27B1) within antigen-presenting cells (APCs). This minimizes the availability of  $1,25(\text{OH})_2\text{D}_3$  and T cell activation. Upon lung injury (2), TLR ligands trigger the release of inflammatory mediators, increasing vascular permeability. Increased TLR activation upregulates MHC and CYP27B1 expression within APCs. The latter generates locally available  $1,25(\text{OH})_2\text{D}_3$  that activates vitamin D receptor (VDR) signaling within T cells to have a plethora of effects. These include efficient TCR signaling within naïve T cells, increased antimicrobial responses, autophagy, and effector cytokine production. As the injury progresses (3), there is a surplus of  $25(\text{OH})\text{D}_3$  and  $1,25(\text{OH})_2\text{D}_3$ , which represses major histocompatibility complex (MHC) expression and dendritic cell maturation, simultaneously decreasing T cell activation as a result. T cells exhibit decreased effector cytokine production and skew toward a more regulatory, pro-resolving response. Once injury begins to resolve (4) and pathogens are cleared, homeostasis is restored, decreasing the available  $25(\text{OH})\text{D}_3$  pool. Remaining  $1,25(\text{OH})_2\text{D}_3$  continues to maintain a regulatory T cell response, while increasing expression of the catabolic enzyme CYP24A1.

pathogenic Th1/Th17 inflammation could prevail, particularly in the presence of environmental insults.

## 18. Summary points

- The capacity of vitamin D to modify effector and regulatory CD4+ and CD8+ T cell function is likely to reflect both direct actions on T cells and indirect effects on APCs.
- $1,25(\text{OH})_2\text{D}$  dampens type 1 and type 17 adaptive T cell responses; data are still emerging on the effects on other T cell subsets, e.g., Tfh, unconventional lymphocyte populations, and T cell plasticity.
- Vitamin D can upregulate type 2 responses; however, there is no evidence that this results in exacerbation of conditions linked to type 2 immunity, such as allergic disease or asthma.
- $1,25(\text{OH})_2\text{D}$  promotes a range of immunoregulatory pathways, including increasing the frequency of Foxp3+Treg and IL-10+ T cells, with good evidence linking experimental findings with in vivo observations.
- T cells can generate  $1,25(\text{OH})_2\text{D}$ ; however, the importance of endogenous production by human T cells versus the requirement for production by other cells within the local milieu remains unclear.
- Vitamin D modulates the function of B cells including immunoglobulin synthesis; however, many effects described in vitro have not been recapitulated in vivo. Vitamin D downregulates IgE synthesis, linked to allergic disease and studies in pediatric cohorts demonstrate an inverse association between vitamin D status and total and allergen-specific IgE. The impact of vitamin D on antibody responses to vaccination remains to be fully elucidated.

- The effects of vitamin D on adaptive immunity are predominantly linked to dampening inflammatory responses, which are likely to underpin observational data that vitamin D deficiency is linked to an increased incidence and poor control of many immune-mediated inflammatory conditions.

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# Vitamin D, microbiota, and inflammatory bowel disease

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## OBJECTIVES

- Present the environmental factors (especially vitamin D and the microbiota) that contribute to the development of inflammatory bowel disease.
- Present the mechanisms by which vitamin D regulates immunity, the microbiota, and experimental inflammatory bowel disease.
- Describe the evidence supporting a role for vitamin D for patients with inflammatory bowel disease.
- Identify the barriers to the use of vitamin D as an adjunct therapy for patients with inflammatory bowel disease.

## 1. Introduction

The Crohn's and Colitis Foundation of America estimates that 3 million Americans have inflammatory bowel disease (IBD). The highest incidence of IBD continues to be in North America and Europe, with the highest percentage of Crohn's disease patients found in Canada [1,2]. However, in the 21st century, IBD has started to occur in Asia, Africa, and South America [2]. Vitamin D deficiency has been linked to the development of immune-mediated diseases, including multiple sclerosis, type 1 diabetes, and IBD [3]. The importance of the commensal microbiota in immune-mediated disease and IBD has been established [4]. In this chapter, the immunoregulatory role of vitamin D and its effect on

the microbiota and the pathology of IBD will be reviewed. The epidemiological evidence connecting vitamin D deficiency to IBD severity, and the data from animal models of experimental IBD will be discussed. Finally, current treatment options for IBD patients will be reviewed, and how vitamin D might be used as an alternative, or a supplemental treatment for patients with IBD.

## 2. What is inflammatory bowel disease?

IBD occurs because of complicated interactions between multiple genetic and environmental factors. Crohn's disease (CD) and ulcerative colitis (UC) are the major forms of IBD. CD and UC are chronic inflammatory disorders of the gastrointestinal tract that are characterized by remitting and relapsing inflammation of the intestinal mucosa [5]. The IBD diseases often result in abdominal pain, diarrhea, and fever. As the disease progresses, rectal bleeding, weight loss, and severe fatigue can occur limiting the quality of life for patients with IBD. Men and women are equally affected by IBD. CD affects teens and young adults with the majority of patients being diagnosed between the ages of 15 and 35 years, and UC affects slightly older individuals; UC patients are usually diagnosed in their mid to late 30s [6]. Because the symptoms of CD and UC are similar, ~10% of patients cannot be definitively diagnosed, and these cases are termed indeterminate colitis. Even though CD and UC share similarities, there are distinct differences in their pathology. The inflammation found in CD most commonly involves the terminal ileum of the small intestine but can be diffuse affecting areas



from the esophagus all the way to the rectum [5,7]. CD is characterized by aggregates or clusters of immune cells, specifically macrophage and T cells that form granulomas; therefore, CD is sometimes described as a granulomatous or granuloma-forming disease [6,7]. Inflammation in CD can be patchy or segmental. These are referred to as “skip” lesions [6,7]. The lesions can become transmural, affecting the entire thickness of the intestinal wall. Unlike CD, inflammation in UC typically involves the rectum and extends proximally in a continuous lesion. Histopathology of UC shows an increase in white blood cells in the lamina propria of the colon and the crypts, which often leads to the development of microabscesses [6,7].

Normally, the body’s immune system protects from invading pathogens such as bacteria, viruses, and fungi but tolerates food antigens and microbes living in the lumen of the intestine. However, in IBD, the immune system is inappropriately activated by the microbes found in the gut. In patients with IBD, the inflammation does not resolve but instead persists. A complicated interplay between genetics and the environment predisposes individuals to the development of IBD. Current research focuses on understanding how the balance between bacteria, the host, and the immune system is maintained, so that new strategies to prevent or treat IBD can be discovered.

### 3. Who gets inflammatory bowel disease?

#### 3.1 Genetic factors

There is clear evidence of a strong genetic component to IBD. IBD is significantly more prevalent within families. In fact, 20%–25% of patients have a close relative with CD or UC [8]. People with a biological relative with IBD are 10 times more likely to develop the disease than the general population and that number increases to 30 times more likely if the relative is a brother or sister [9]. Advances in genetics research have led to the discovery of several IBD susceptibility genes [10].

Single-nucleotide polymorphisms (SNPs) in genes of the immune system are prevalent in IBD [11]. The major histocompatibility complex (MHC) is expressed on all nucleated cells and controls the generation of antigen-specific immunity. MHC genes regulate the ability of the immune response to distinguish between self-antigens and foreign antigens. In immune-mediated diseases including MS, rheumatoid arthritis, and IBD, the immune response inappropriately targets host tissues. SNPs in MHC genes have been described in patients with these immune-mediated diseases. SNPs in MHC affect the nature of the immune response and are associated with IBD [11].

Additional IBD-associated genes have been identified that regulate immune cell development, function, or activation [10]. Some genetic polymorphisms found in patients with IBD affect the function of the immune system by altering the immunomodulatory cytokines produced by it. Interleukin (IL)-10 is an important suppressive cytokine, and SNPs in and around the IL-10 gene have been associated with UC [12]. SNPs in inflammatory cytokine genes or in genes for receptors of inflammatory cytokines, including tumor necrosis factor (TNF)- $\alpha$ , IL-8, IL-12, IL-18R, and IL-26, have been described in patients with CD and UC [11–14]. Several SNPs in genes associated with IBD play a role in the development of inflammatory T cells. Subsets of these genes encode cytokines or cytokine receptors that affect Th17 differentiation (IL-23 receptor) [11]. SNPs have been described that can affect or disrupt the IL-23R signaling cascade including STAT3 and STAT4 (associated with CD and UC, respectively) and JAK2 (associated with both) [15]. Another IBD susceptibility gene is CTLA4, which can suppress T cell activation [16]. An important IBD susceptibility gene is nucleotide oligomerization domain (NOD)2 (also known as CARD15) [10]. SNPs of NOD2 have been shown to limit the immune system’s ability to recognize bacteria [11]. NOD2 encodes a cytosolic microbial molecular pattern recognition receptor (PRR) that belongs to a large group of innate immune receptors [6]. Like NOD2, NOD1 is also a PRR, but NOD1 SNPs are more strongly associated with UC than CD [17]. Toll-like receptor (TLR) 4 recognizes bacterial lipopolysaccharides and helps activate the immune response to invading microbes. Polymorphisms in the gene encoding TLR4 have been shown to be associated with both CD and UC [18]. In each of these cases, failure to recognize bacteria properly may result in an abnormal immune response to commensal microflora.

Other IBD susceptibility genes have roles in maintaining intestinal integrity. Extracellular matrix protein 1 is implicated in the interaction between the intestinal epithelium and the basal membrane, and SNPs in the gene encoding this protein have a strong association specific for UC [12]. NOD-like receptor protein 3 SNPs are associated with CD, and in mouse models, loss of this protein has been shown to result in the loss of epithelial integrity [19,20]. Loss of intestinal integrity may result in systemic infiltration of commensal flora and increased inflammation in the underlying tissue.

To date, over 500 SNPs have been discovered in genes of the vitamin D pathway with 470 of the SNPs being found within the VDR gene [21]. The VDR gene maps to a region on chromosome 12 that has been functionally linked to IBD by genome-wide association [22]. Through the use of genome-wide association studies, polymorphisms in the VDR gene were shown to increase

susceptibility to CD and UC [22,23]. A meta-analysis of studies done before 2014 showed that there is one polymorphism in the VDR that is associated with higher risk of CD and one VDR polymorphism associated with a lower risk of UC [24]. Additional studies are needed to determine the extent of VDR polymorphisms in patients with IBD and whether the polymorphisms result in functional outcomes in vitamin D signaling.

1,25-Dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ), the hormonal form of vitamin D, is known to be a transcriptional regulator that targets genes via interaction in trans with vitamin D response elements (VDREs). Several IBD-associated genes have VDREs. Cytokine genes such as IL-2, IFN- $\gamma$ , IL-12, and others are transcriptional targets of  $1,25(\text{OH})_2\text{D}$  [25]. Sequence analysis of human MHC class II genes revealed VDREs in the promoter [26,27]. The IBD-associated gene NOD2 is regulated by vitamin D and has an enhancer VDRE [28]. Vitamin D is a transcriptional regulator of several genes important in controlling the immune response and the development of IBD.

Other pathways that control vitamin D function, beyond the VDR polymorphisms, have not been examined in relation to IBD. There is evidence that SNPs within the gene encoding the vitamin D- $1\alpha$ -hydroxylase (*CYP27B1*) or the vitamin D 25-hydroxylase (*CYP2R1*) that is required for production of  $1,25(\text{OH})_2\text{D}$  are associated with MS [29,30]. SNPs in the genes of other components of the vitamin D pathway have been described including genes for vitamin D catabolism and the vitamin D-binding protein [21]. The effects of vitamin D and its metabolites could be regulated by alterations in genes that affect vitamin D transport, metabolism, catabolism, or function. To date, these genes have not been studied in IBD patients.

### 3.2 Environment

Genetic polymorphisms account for only 10%–20% of the overall risk in CD and even less for UC [31]. The concordance rate for IBD development in genetically identical twins is only 20% in UC and 50% in CD [3]. These findings indicate that important environmental factors affect the development of IBD.

IBD is largely a disease of the developed world, with the majority of cases being reported in North America and Europe. IBD occurs more often in urban than in rural areas, and within the northern hemisphere, IBD is more prevalent in northern versus southern climates [2,32]. The prevalence of IBD worldwide and specifically in countries that were previously considered “low-incidence areas,” such as Japan and India, is also increasing [2]. The increased prevalence of IBD cannot be explained by genetics. First- and second-generation

immigrants from “low-incidence areas” that have moved to countries with higher incidences adopt risk levels similar or higher than the residents of that country [11].

The environmental factors that may play a role in IBD are poorly defined and may be numerous. The incidence of IBD is higher in industrialized nations and even more so for urban areas within those nations. Environmental factors that may be different in IBD low versus high areas include the following: diet, lifestyle, pollution, exposure to potentially harmful chemicals, and exposure to smoke. Smoking tobacco is one of the most highly associated risk factors for CD but interestingly has been shown to be helpful in patients with UC [33].

One critical environmental factor associated with IBD is the microbiota. Bacterial exposure can be shown to either induce or prevent experimental IBD. In one animal model of IBD, germ-free mice are protected and in another experimental model germ-free mice develop more severe disease [12,34]. Infection with *Mycobacterium avium paratuberculosis* and several species of *Helicobacter* is associated with IBD [35,36]. The causative and/or protective role of the microbiota has been demonstrated by microbial transplants into germ-free or antibiotic-treated animals that result in changes in the severity of experimental colitis [37,38]. Microbial transplants have also been done in humans with some success, especially in UC patients [39–41]. The composition of the microbiota is of critical importance for health, and dysbiosis is linked to the development of IBD.

### 3.3 Microbiota in health and disease

A population of nearly 100 trillion dynamic and diverse microbiota—between 500 and 1000 different species—inhabit the human gut [42]. The gut microbiota is essential for normal immune system development, displacement of pathogens, and extraction of additional energy (e.g., short-chain fatty acids) from otherwise non-digestible dietary substrates [43–45]. Highlighting the importance of the gut microbiota, numerous human metabolic diseases and conditions have been attributed to significant alterations in the microbiota [44]. For example, IBD is associated with less diversity in microbiota and an expansion of opportunistic pathogens that cause severe intestinal inflammation [44,46]. The composition of the microbiota also regulates health and diseases outside of the gastrointestinal (GI) tract including multiple sclerosis, cancer, and neurological diseases [47,48]. Regulation of the microbiota is important for the maintenance of health and homeostasis.

The human infant is largely germ-free until after birth. As babies are born microbiota, antibodies and nutrients are transferred from the mother to the neonate

[49,50]. Maternal antibodies help to protect the neonate from infection as the baby develops [51]. The microbial colonization of the neonate plays a critical role in the expansion of innate immune cells, development of intestinal epithelial cells, and establishment of a complex and diverse microbiota [50]. Evidence suggests that the changes in the microbiota that occur early in life can persist and may partially explain increased risk for disease later in life [52,53]. Over the lifespan of individuals, the microbiota continues to change with age, diet, and antibiotic use.

Acquired immunity requires the microbiota. Adult germ-free mice have very small lymph nodes and low levels of IgA, IgM, IgG antibodies, and very high IgE levels [54]. The elevated IgE in the germ-free mice was shown to be a result of elevated IL-4 and increased type 2 responses [54,55]. Colonization of the germ-free mice with microbiota reduced IL-4 secretion and down-regulated the IgE response [54,56]. Colonization of mice with intact microbiota was shown to induce type 1 T helper (Th1) T cells, Th17, and regulatory T cells (Treg) in the GI tract [57]. A key microbe for the induction of Th17 cells in the GI tract was shown to be segmented filamentous bacterium [57]. Microbe-specific regulatory T cells (FoxP3/ROR $\gamma$ t+) have been described in the colon of mice that help in maintaining immune tolerance and the balance between pro- and antiinflammatory responses [55,58]. Microbial transplantation into germ-free mice reconstituted the FoxP3/ROR $\gamma$ t T reg cells and demonstrated their dependence on the microbiota [55,58]. *Bacteroides* and *Clostridium* species are among the bacteria that induce T reg cells [59,60]. T and B cell homeostasis depend on the commensal microbiota.

Changes in the diet impact the microbiota. Dietary change resulted in a rapid but reproducible change in the microbiota of mice [37,61]. Experimentally in mice, the diet affected the host microbiota more so than genetics [61]. Beginning with the choice to breastfeed or formula-feed, the effect of diet on the human microbiota has been demonstrated [52,53,62]. The Mediterranean diet and the Western diet are two extremes that have been shown to have opposing effects on the development of chronic disease [62]. The key differences between the Mediterranean versus Western diets are the lower amounts of animal fats/protein, lower sugar, higher and more varied fiber, and higher plant protein. Feeding Western diets to mice resulted in a shift in the microbiota within 3.5 days [61]. The microbiota of the mice fed Western diets had reproducible increases in Firmicutes and Verrucomicrobia and decreased Bacteroidetes phyla [61]. Obesity, which is associated with the consumption of western diets, resulted in higher Firmicutes/Bacteroidetes ratios [63]. The Firmicutes/Bacteroidetes ratio of individuals who adhered to a Mediterranean diet were lower than those who did not

in the PREDIMED (PREvencion con Dieta MEDiterranea) study [64]. In addition, the participants that had eaten a Mediterranean diet had higher Bacteroidetes phyla [64]. Another indication of adherence to the Mediterranean diet was an increase in short-chain fatty acids produced by the microbial metabolism of fiber [64]. The health-promoting effects of the Mediterranean diet and the disease-inducing effects of Western diet have profound effects on the commensal microbiota.

Manipulation of the microbiota is not limited to macronutrient intakes. In mice, changing or adding a single component to the diet alters the microbiota [37,65]. White button mushroom feeding altered the microbiota and improved glucose sensitivity compared with the control fed mice [66]. Single micronutrient deficiencies, including vitamin A and vitamin D deficiency, shift the composition of the microbiota [38,67,68]. There is a dominant effect of diet on the microbiota of an individual. The efficacy of interventions, such as microbial transplantation or probiotics, to shift the microbiota toward health promotion, will be affected by the diet. Approaches that seek to manipulate the microbiota would need to include lifestyle diet changes to sustain the healthy microbiota. Whether single nutrient or other dietary interventions can cause stable and long-term shifts in the microbiota to promote health is an open question.

### 3.4 The “hygiene hypothesis”

The higher incidence of IBD in industrialized countries has led to the proposal of the “hygiene hypothesis.” The hygiene hypothesis suggests that because of the use of vaccines, improved sanitation, and reduced rates of infection, the immune system does not receive critical signals, which impact the development of the immune system and result in increased incidence of immune-mediated disease [69]. The immune system overreacts and subsequently fails to shut down inflammation [32]. Rural areas have fewer cases of IBD. Several studies have shown that children who live on farms have a lower prevalence of immune-mediated diseases including asthma, allergies, and IBD [32,69]. Data indicate that elements of the farm lifestyle may expose children to factors that activate the immune response but do not cause inflammation. Possibilities include exposure to endotoxins, contact with animals or soil, microbial exposure, and diets rich in dairy products [32,69]. Interestingly, in 2007, a case-controlled study showed that exposure to farm animals, especially cattle, during the first year of life had a protective effect against developing IBD [70].

Improved hygiene in developed countries limits exposure to previously ubiquitous infectious agents such as several different types of worm (helminth)

infection. Helminths are parasites that infect humans throughout the world and are thought to play an important immunoregulatory role in the intestine. The response of the immune system to helminths, such as *Schistosoma mansoni* and *Trichinella spiralis*, has been shown to be protective against experimental models of IBD and in patients with IBD [11]. In addition, infection with these organisms results in reduced inflammation and increased production of mucins and water secretion into the lumen of the gut, which also reduces inflammation [32]. Epidemiological data suggest that helminth infection is inversely correlated with the economic status of the region as well as incidence of IBD and other immune-mediated diseases [11].

### 3.5 The “vitamin D hypothesis”

The vitamin D hypothesis has been proposed and suggests that vitamin D status may be an environmental factor involved in the development of IBD [71,72]. A major source of vitamin D comes from a photolysis reaction in the skin after exposure of skin to sunlight. Skin pigment, aging, time of day, season, and latitude dramatically affect vitamin D synthesis [73]. The incidence of IBD is higher in more northern regions of the United States and Canada. Vitamin D status is especially low during winter months in areas with the greatest seasonal fluctuation [73,74]. Many of the environmental factors that are present in high-risk areas for IBD would also result in decreased availability of vitamin D. Factors such as air pollutants and decreased outdoor activity are known to reduce vitamin D synthesis [73]. Intentional avoidance of sunlight exposure of our skin also reduces vitamin D production in the skin. In developed countries, people limit skin exposure to sunlight to avoid skin cancer. The use of high SPF sunscreens not only decreases the risk of skin cancer but also reduces the amount of vitamin D made in the skin [73].

The other source of vitamin D is the diet. Most diets are limited in natural sources of vitamin D. Vitamin D–fortified dairy and grain products, egg yolk, and ultraviolet B-exposed mushrooms contain some vitamin D [75]. Oily fish such as salmon or cod are high natural sources of vitamin D [75]. Obesity results in lower serum levels of 25-hydroxyvitamin D (25(OH)D) [76], and obesity in IBD patients is associated with increased severity of IBD [77].

Vitamin D insufficiency (25(OH)D 20–35 ng/mL) and deficiency (<20 ng/mL) are common in northern regions of the northern hemisphere, and several studies report an even higher prevalence of insufficiency and deficiency in adult and pediatric patients with IBD [78]. Vitamin D deficiency is common even when the patient is in remission [78,79]. Several biomarkers

indicative of inflammation (fecal calprotectin and IL-6) have been shown to be inversely associated with vitamin D status [80,81]. Conversely, the antiinflammatory cytokine IL-10 was higher with increased serum 25(OH)D levels [82]. Vitamin D deficiency was associated with high prevalence of osteoporosis, osteopenia, and low bone mineral density (BMD) in patients with IBD [83]. Similarly, IBD patients have a higher rate of bone fracture [84]. These trends may be a consequence of the disease, and/or glucocorticosteroid treatment that has been shown to decrease BMD. Many newly diagnosed IBD patients have reduced BMD, and the increased IBD prevalence is slightly higher in patients with CD than UC [85]. Low serum 25(OH)D levels are a common finding in patients with IBD and may both predispose and exacerbate IBD that contributes to the development of bone disease.

Several cross-sectional and observational studies have been performed (Table 97.1; [96]) to investigate the association between the severity of UC or CD and serum 25(OH)D levels (Table 97.1). Patients with vitamin D deficiency had more severe UC [86–91]. Vitamin D insufficiency led to relapses that were closer together than in vitamin D–sufficient UC patients (Table 97.1; [87,88]). Vitamin D status was associated with improved quality of life for UC patients [86,90]. Vitamin D deficiency or vitamin D insufficiency was associated with higher CD disease and longer CD duration than vitamin D–sufficient CD patients (Table 97.1; [89,91–95]). Multiple studies establish an inverse correlation between vitamin D status and the severity of both UC and CD.

### 3.6 The gut epithelium, commensals, and inflammatory bowel disease

The gastrointestinal tract and the mucosal immune response form a barrier designed to allow nutrient and water absorption but not systemic infection with the microbes harbored in the gut. Maintaining the mucosal barrier function of the gastrointestinal tract is critical, and patients with IBD have increased permeability of the small intestine and colon that leads to malabsorption and systemic exposure to microbes [97]. Expression of tight junction proteins such as claudin-1, ZO-1, occludin, and E cadherin by epithelial cells in the gastrointestinal tract maintains epithelial integrity [97]. Experimental models of IBD show increased gastrointestinal tract permeability; as a result, the animals have leaky guts [98,99]. Compromised barrier function in both humans and experimental animals is linked to inflammation, colitis, and IBD.

IBD is associated with less diversity in microbiota, dysbiosis, and an expansion of opportunistic pathogens



**TABLE 97.1** Associations between serum 25(OH)D levels and clinical IBD severity.

Parameter <sup>a</sup>	Serum 25(OH)D <sup>b</sup>			Reference(s)
	Deficient (<20 ng/mL)	Insufficient (20–35 ng/mL)	Sufficient (35–80 ng/mL)	
UC severity	+++	+	+	[86–91]
UC duration	+++	+	+	[87]
UC relapse	ND	++	+	[88]
UC quality of life	ND	+	–	[86,90]
CD severity	+++	++	–	[89,91–95]
CD duration	++	+	–	[91]

<sup>a</sup>Parameter and the association with IBD disease: –, remission; +, mild disease; ++, moderate disease; +++, severe disease.

<sup>b</sup>A range of cutoffs were used to define the 25(OH)D level between vitamin D insufficiency and sufficiency: 30, 32, and 35 ng/mL of 25(OH)D. UC, ulcerative colitis; CD, Crohn's disease; ND, not determined.

that cause severe intestinal inflammation [100,101]. Patients with IBD had fewer commensal from the phyla Firmicutes and Bacteroidetes and higher potentially pathogenic Proteobacteria and Actinobacteria phyla members [101]. In particular, bacteria that ferment non-digestible dietary fiber into butyrate are lower in IBD patients as compared with healthy controls [101]. Nonbacterial members (viruses and fungal microbes) of the microbiota have also been described as being in dysbiosis in IBD patients [102,103]. The composition of the gut microbiota is critical for the maintenance of gastrointestinal homeostasis. Dysbiosis of the bacteria, viruses, and fungi is associated with the development of IBD.

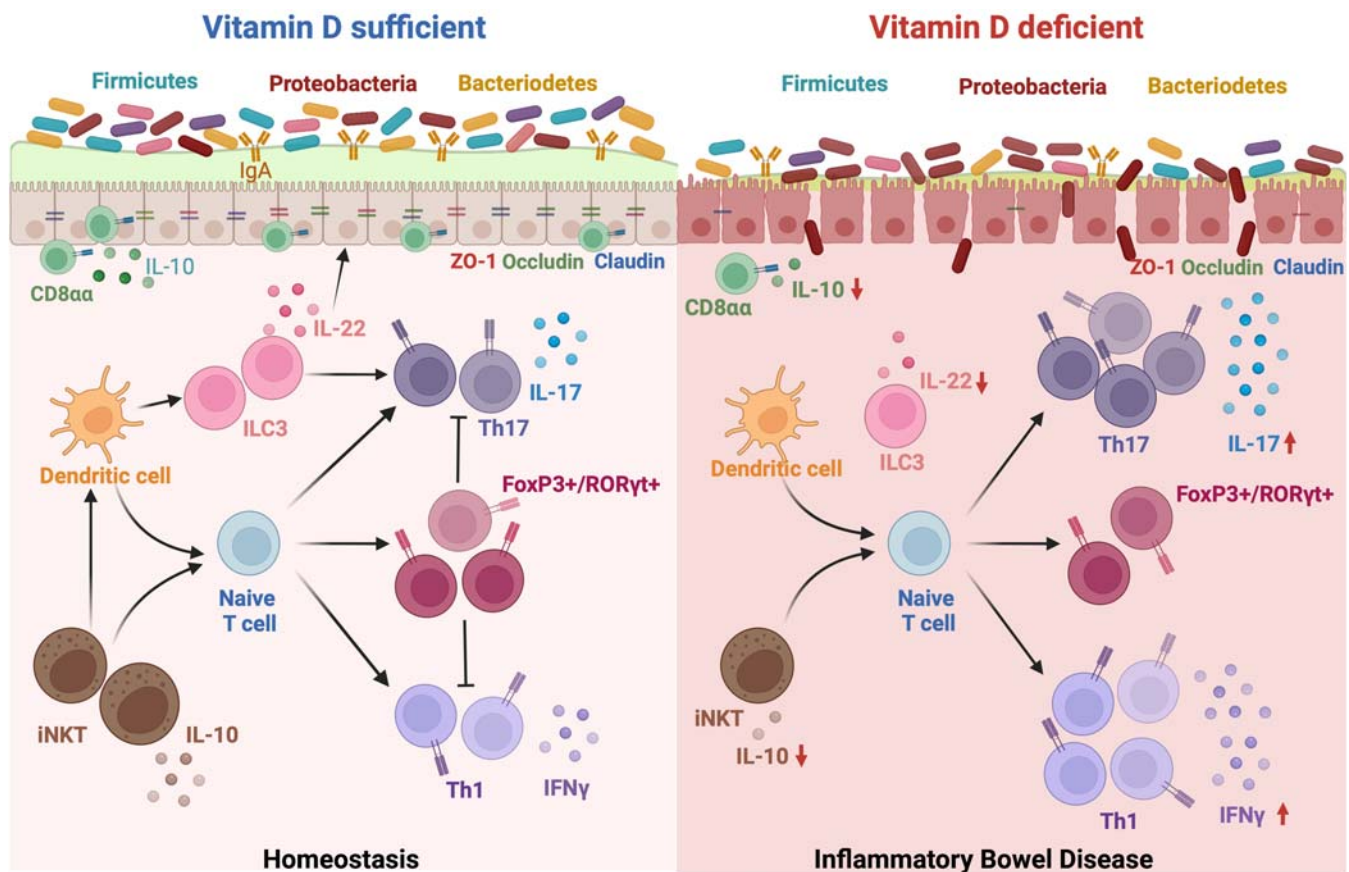
#### 4. The immune system and inflammatory bowel disease

The gastrointestinal immune system is a population of heterogeneous cells whose role is to maintain tolerance of the large number of antigens present in food as well as the abundant microbiota. The mechanisms by which tolerance in the gut is controlled includes complex interactions between the microbiota, the innate, and adaptive immune systems.

In healthy intestine, the epithelium provides a tight barrier and interacts with the commensal microbiota at the mucosal interface (Fig. 97.1). Vitamin D receptors are expressed constitutively in epithelial cells found in the small intestine and colon, and the deletion of the VDR in the gut epithelium resulted in an impaired barrier and increased susceptibility to experimental colitis [104]. Vitamin D and 1,25(OH)<sub>2</sub>D induce the expression of several different proteins (ZO-1, claudins, and E-cadherin) important for the integrity of the gut barrier [104–106]. The gut is largely tolerant of the commensal

bacteria and does not mount an immune response to it. Injury of the gut results in impaired intestinal permeability and dysbiosis of the microbiota with a shift in the microbiota that favors pathogens such as the Proteobacteria phyla members (Fig. 97.1; [107]). Following injury, including gastrointestinal infection, the mucosal immune system mounts an immune response to clear the infection and then reinstates homeostasis. The innate immune system is the first line of defense against an invading pathogen. Innate immune cells such as macrophage and dendritic cells (DCs) recognize pathogens via pattern recognition receptors (TLRs or NOD-like receptors) and become activated. Activated cells then become antigen-presenting cells; they process peptide components of the pathogen and present these antigen peptides to T cells in the local environment. The type of T helper (Th, CD4<sup>+</sup> T cell) response that is generated will dictate the outcome of the infection.

Different T cells are required to induce protection from different pathogens. Effector T cell responses including Th1 and Th17 cells are required to fight many different gastrointestinal infections. In IBD uncontrolled Th1 and Th17, immune responses occur (Fig. 97.1) and strategies to suppress the Th1 and Th17 cells effectively suppress experimental IBD. Patients with IBD often have increased Th1 cells in the intestine that produce high levels of the Th1 inflammatory cytokine IFN- $\gamma$  [108]. Th17 cells produce IL-17 that has been shown to play a pathogenic role in several immune-mediated diseases including MS, type 1 diabetes, and IBD [109,110]. Clearance of enteric infections requires the induction of antigen-specific Th17 cells [111]. The IL-17 produced at the peak of infection with enteric pathogens has been used to model the GI inflammation that occurs with enteric infection [111]. Resolution of the GI inflammation following infection requires the inhibition of Th17 cells and the induction



**FIGURE 97.1** Vitamin D maintains homeostasis of the gastrointestinal tract. Vitamin D sufficiency and the healthy gastrointestinal tract: The mucosal epithelium forms a barrier to prevent infection with the microbes found in the gut. The healthy gastrointestinal tract harbors a diverse population of microbes. The microbes in the vitamin D-sufficient gut induce FoxP3<sup>+</sup>/RORγt<sup>+</sup> T reg cells. The vitamin D-sufficient intestinal epithelial cells express ZO-1, occludin, and claudin that are important tight junction proteins required for an intact barrier. Vitamin D supports ILC3 cells that produce IL-22 important for maintaining barrier integrity, responding to microbial antigens and regulating T cell responses. With the participation of dendritic cells, naïve Th cells require vitamin D to generate enteric specific Th17 cells. In the healthy intestine, there is a balance between effector T cells (Th1 or Th17) and regulatory T cells (CD8αα, Treg, and iNKT cells) that prevent the host from generating an immune response to the commensals, while protecting from gastrointestinal infection. The gut epithelium during vitamin D deficiency is impaired, and dysbiosis occurs that breaches the mucosal barrier. The microbiota in the vitamin D-deficient host has increased numbers of proteobacteria, increased firmicutes/bacteroidetes ratios, and a decrease in the diversity of the microbes in the gut. The vitamin D-deficient gut has fewer commensal microbiota that can induce FoxP3<sup>+</sup>/RORγt<sup>+</sup> T reg cells. The dysbiosis and shift toward pathogenic bacteria leads to increased DC activation, antigen presentation, and cytokine production. Infection-induced Th17 cells are slow to develop in the vitamin D-deficient host. Overproduction of IL-17 and IFN-γ occurs in the vitamin D-deficient host. In addition, there are few regulatory cells and less IL-10 in the vitamin D-deficient gut. Too few iNKT cells and CD8αα T cells result in the inability to turn off the Th17 and Th1 cell response, and as a result, inflammation and colitis develop. IFN, interferon; ILC, innate lymphoid cell; IL, interleukin; iNKT, invariant natural killer; Th, T helper cell; Treg, T regulatory cell. Created with BioRender.com.

of Treg cells (Fig. 97.1; [111,112]). Some patients with UC have an increased Th2 cell response that is characterized by the production of IL-4, IL-5, and IL-13 instead of the Th1 and Th17 cell cytokines [113,114]. In addition, CD patients that are anti-TNF-α nonresponders had Th2 cells in the lamina propria [113]. Uncontrolled Th1, Th17, or Th2 cell responses are associated with both UC and CD.

There are a number of other types of T cells that play a role in turning off (regulating) immune responses in the gut (Fig. 97.1). The invariant natural killer T (iNKT) cells produce cytokines early during an immune response

acting on the DC and influencing the development of the T cell responses. iNKT cells in the intestine become activated by the epithelial cells to produce inhibitory cytokines such as IL-10 [115]. iNKT cells are early producers of many cytokines including IFN-γ, IL-4, IL-13, and IL-10 [115]. The type of cytokines produced by the iNKT cells dictates the nature of the resulting T cell response [115]. Activation of iNKT cells protects mice from experimental IBD, and reduced numbers of iNKT cells have been shown in experimental MS and type 1 diabetes [116]. IBD patients have decreased numbers of iNKT cells [117]. FoxP3<sup>+</sup> CD4 T cells (Treg) act to inhibit

the proliferation of T cell responses both in vitro and in vivo [118]. Animals that lack Treg cells spontaneously develop experimental IBD [12]. The spontaneous development of IBD in mice that lack Tregs is because of the inability to turn off T cell responses to the commensal flora [12]. T regs in the GI tract can be FoxP3+ or FoxP3+/ROR $\gamma$ t+ [119]. In the colon lamina propria, most of the Tregs are FoxP3+/ROR $\gamma$ t+ and require microbiota since they are largely absent in germ-free mice [55]. Tregs have been shown to induce programmed cell death or apoptosis in effector T cells (Th1 and Th17 cells), suppress proliferation, and produce the suppressive cytokines IL-10 and TGF- $\beta$ 1 [118]. Transfer of Tregs has been shown to suppress experimental IBD in vivo [59]. iNKT cells and Tregs downregulate and control the Th1 and Th17 responses in the gastrointestinal tract (Fig. 97.1).

The normal vitamin D-sufficient gut-associated lymphoid tissue harbors specialized gut-specific T cells that also have a regulatory function. These unique T cells express a homodimeric form of CD8-CD8 $\alpha\alpha$ ; the CD8 $\alpha\alpha$  on the T cells acts to dampen signals coming into the cell through the T cell receptor [120]. CD8 $\alpha\alpha$  is expressed on T cells in the gut to prevent them from responding to the bacteria and food antigens found there (Fig. 97.1; [121]). In addition, CD8 $\alpha\alpha$  T cells in the intestinal epithelium can also suppress inflammation by producing suppressive cytokines such as IL-10 and TGF- $\beta$ 1 [122,123]. Adoptive transfer of CD8 $\alpha\alpha$  T cells has been shown to suppress experimental IBD [122]. Unique T cells that express CD8 $\alpha\alpha$  are regulatory cells in the gut.

#### 4.1 Vitamin D regulates T cell responsiveness

As discussed elsewhere in this volume, the VDR is expressed in all immune cells that have been examined. The level of VDR expression in certain types of immune cells including T cells increases after activation [124]. Vitamin D deficiency or VDR knockout (KO) mice have been shown to have increased susceptibility to several different experimental models of IBD, including infection-induced colitis [125–127]. In addition, the high-affinity ligand for the VDR, 1,25(OH) $_2$ D, suppresses experimental models of immune-mediated disease including IBD [128,129].

Treatment with 1,25(OH) $_2$ D in vitro has been shown to inhibit differentiation and activation of DC [130]. 1,25(OH) $_2$ D-treated DC stay in a more immature state characterized by reduced antigen presentation, decreased IFN- $\gamma$  production, and increased production of IL-10 [131,132]. Macrophage proliferation and differentiation is also inhibited by 1,25(OH) $_2$ D in vitro, and treatment of the macrophage after activation inhibits

the production of IL-12 and TNF- $\alpha$  [133,134]. These effects on macrophage and DC result in reduced Th1 cell activation.

T cells are also direct targets of 1,25(OH) $_2$ D. Purified T cells activated and 1,25(OH) $_2$ D $_3$  treated had lower proliferation and less IL-2 and IFN- $\gamma$  production [135,136]. Conversely, CD4 $^+$  Th cells from VDR KO mice overproduce IFN- $\gamma$  and proliferate twice as quickly in mixed lymphocyte reactions [125]. Treatment of peripheral blood mononuclear cells from IBD patients with physiological levels of 1,25(OH) $_2$ D decreased the production of IFN- $\gamma$  [137]. IL-4 inhibits Th1 cells, and IFN- $\gamma$  inhibits Th2. Treatment of either human or mouse T cells and iNKT cells in vitro with 1,25(OH) $_2$ D induced IL-4 production [138]. Several groups have shown that 1,25(OH) $_2$ D treatment enhances the production of IL-4 and IL-5 and augments the expression of the Th2-specific transcription factor GATA-3 [139,140]. 1,25(OH) $_2$ D inhibits Th1 and induces IL-4 production from Th2 and iNKT cells.

Th17 cells are also targets of 1,25(OH) $_2$ D. 1,25(OH) $_2$ D treatment of DC reduces Th17-inducing cytokines IL-6 and IL-23 [141]. Treatment of CD4 $^+$  Th cells with 1,25(OH) $_2$ D under Th17-polarizing conditions (e.g., under the influence of IL-6 and TGF- $\beta$ 1) reduces the number of Th17 cells that develop [142]. VDR KO and vitamin D-deficient CD4 T cells overproduced IL-17 [143]. In addition, transfer of VDR KO CD8 $^+$  T cells to immunodeficient mice resulted in the rapid expansion of naïve CD8 $^+$  T cells that homed to the gut, produced IL-17, and induced IBD symptoms [144]. Enteric infection of vitamin D-deficient mice resulted in the delayed clearance of the infection that coincided with a delay in the generation of Th17 cells [127]. The induction of IL-22 and the inhibition of IL-17 help to resolve inflammation in the vitamin D-sufficient host [127]. Human peripheral blood mononuclear cells from IBD patients overproduce IL-17, and treating them with a vitamin D analog reduces the production of Th17 cells [145]. Vitamin D supports the induction of Th17 responses following infection. As the infection is controlled, vitamin D inhibits IL-17.

Vitamin D has been shown to control regulatory T cells including iNKT cells, Treg cells, and CD8 $\alpha\alpha$  T cells (Fig. 97.1). Expression of the VDR is required for normal development and function of iNKT cells [146]. 1,25(OH) $_2$ D increases iNKT cell cytokine production but has no effect on the numbers of iNKT cells [146]. VDR KO mice essentially have no functional iNKT cells [146]. The absence of iNKT cells is partially responsible for the susceptibility of VDR KO mice to dextran sodium sulfate (DSS)-induced colitis [147]. The ability of 1,25(OH) $_2$ D to suppress several experimental autoimmune diseases occurs via the induction of Treg cells [148]. 1,25(OH) $_2$ D has been shown in vitro to increase



Treg numbers [131,149]. However, VDR KO mice have normal numbers of thymic-derived Treg cells but fewer inducible Tregs [143]. Microbiota inducible T regs (FoxP3+/ROR $\gamma$ t+) in the colon are vitamin D targets. The FoxP3+/ROR $\gamma$ t+ T regs were fewer in the colons of D– than D+ mice [67]. Microbial transplants from D+ and D– mice into germ-free mice phenocopied the Treg cell differences in the recipients of D+ or D– microbiota [67]. The CD8 $\alpha\alpha$  T reg cells also require vitamin D for normal development. Vitamin D–deficient and VDR KO mice have half as many of the CD8 $\alpha\alpha$  T cells as their WT counterparts (Fig. 97.1; [150]). In addition, the CD8 $\alpha\alpha$  T cells from VDR KO mice are functionally impaired; they produce less IL-10 than those from the WT mice [150]. 1,25(OH) $_2$ D enhances the development and function of iNKT cells, Tregs, and CD8 $\alpha\alpha$  T cells. In the absence of vitamin D or the VDR, T regs, iNKT cells, and CD8 $\alpha\alpha$  T cells are fewer resulting in unregulated inflammation in the GI tract.

In summary, the vitamin D system (25(OH)D status, 1,25(OH) $_2$ D, and VDR expression) influences both the innate and acquired immune system in the gut. Vitamin D is essential for the generation of Th17 cells in the colon following enteric infection (Fig. 97.1). Following injury or infection, 1,25(OH) $_2$ D directly and indirectly inhibits pathogenic Th1 and Th17 cell responses (Fig. 97.1). In addition, vitamin D is required for iNKT cells, Treg cells, and CD8 $\alpha\alpha$  T cells to further suppress inflammation in the gastrointestinal tract (Fig. 97.1). When vitamin D is low, there is a limiting amount of 1,25(OH) $_2$ D, and therefore the VDR does not function. Low vitamin D leads to an absence of regulatory T cells and overactive Th1 and Th17 responses, resulting in excessive inflammation in the gastrointestinal tract and IBD (Fig. 97.1).

## 5. Experimental models of inflammatory bowel disease

Much of our knowledge about the mechanisms involved in IBD comes from experimental animal models of intestinal inflammation [111,151]. Although these models provide insight into the pathogenesis of IBD, there is not a mouse model that completely mimics either CD or UC. The aim of these models is to provide tools to researchers that allow for investigation into specific aspects of the diseases. There are several different animal models of IBD. Some experimental IBD models result spontaneously following KO of regulatory cytokines or cells, whereas others are induced following chemical injury, infection with enteric pathogens or immunization. In most of the models, there is interplay between the inability to control inflammation in the gut, the types and complexity of the bacteria in the gut, and loss of barrier function. The IL-2 KO mouse model

is unique in that germ-free mice develop colitis and are one example of an IBD model where the composition of the microbiota does not influence development of colitis [151]. Examination of several different experimental models of IBD can give a more comprehensive view of the effects of vitamin D on several important characteristics of the human disease.

Deletion of the suppressive cytokine IL-10 results in spontaneous intestinal inflammation. The intestinal inflammation that develops in IL-10 KO mice is because of an uncontrolled immune response to the commensal microflora in the intestine [12,152]. IL-10 KO mice that are either vitamin D deficient or VDR/IL-10 double KO develop severe fulminating colitis that is characterized by epithelial hyperplasia, significant weight loss, and premature mortality (Table 97.2; [125]). The severity of the inflammation is associated with an increase in the Th1 response to the bacteria in the intestine including increased IFN- $\gamma$ , IL-12, TNF- $\alpha$ , and IL-1 $\beta$  [152]. In contrast, feeding supplemental 1,25(OH) $_2$ D prevents inflammation in IL-10 KO mice and, when given after the onset of intestinal inflammation, can block the progression of the disease by reducing TNF- $\alpha$  through inhibition of several genes of the TNF- $\alpha$  pathway (Table 97.2; [128,129,157]).

A T cell-driven model of intestinal inflammation develops when naïve CD4 T cells are transferred to mice that lack T and B cells (recombination activating gene (Rag) KO). Rag KO mice that receive the transferred T cells develop a wasting disease as a result of increased inflammation in the small intestine and colon that is driven by uncontrolled Th1 and Th17 responses [158]. Transferring naïve VDR KO CD4 T cells to Rag KO mice increases the severity and induces a more rapid onset of the disease than WT CD4 T cells (Table 97.2; [125]). In addition, transfer of VDR KO CD8 $^+$ , but not WT CD8 $^+$  T cells, to Rag KO mice resulted in accumulation of disease inducing CD8 cells that produced IL-17 and IFN- $\gamma$  (Table 97.2; [144]). Cotransfer of T regs that were either FoxP3+ or CD8 $\alpha\alpha$ + suppressed IBD in these T cell transfer models [122,159]. VDR KO FoxP3+ Treg cells are as good as WT FoxP3+ Treg at suppressing IBD in the Rag KO transfer model [150]. Naïve VDR KO T cell transfer–induced colitis is more severe than colitis when naïve WT T cells are transferred.

Infection with *Citrobacter rodentium* (*C. rodentium*) has been used as a model of infection-induced colitis [111]. Host resistance to *C. rodentium* infection depends on early innate lymphoid cells (ILC)-3 cells that produce IL-22 and induce Th17 cells that clear the infection [111]. Following the induction of Th17 cells that clear the infection, FoxP3+/ROR $\gamma$ t+ T regs resolve inflammation. Vitamin D–deficient and VDR KO mice developed severe *C. rodentium* infection and colitis (Table 97.2; [127,155]). Vitamin D–insufficient mice



**TABLE 97.2** Vitamin D and experimental inflammatory bowel disease.

Model	Immune system <sup>a</sup>	Vitamin D Deficiency <sup>b</sup> /VDR KO	1,25(OH) <sub>2</sub> D	Germ-free [151]
DSS [38,106,147,153]	Innate	+++	+	+++
TNBS [141]	Innate	ND	+	ND
IL-10 KO [125,129,152]	Th1/Th17/Treg deficient	+++	++	—
IL-2 KO [154]	Th1/Th17/Treg deficient	ND	+++	+++
T cell transfer [125,144,150]	Th1/Th17/CD8	+++	+	—
<i>Citrobacter rodentium</i> [127,155,156]	Th17	+++	+	ND

<sup>a</sup>Disease-causing immune cells.<sup>b</sup>Disease severity with vitamin D deficiency/VDR KO, 1,25(OH)<sub>2</sub>D treatment or germ-free mice: —, no disease; +, mild disease; ++, moderate disease; +++, severe disease; ND, not determined.

DSS, dextran sodium sulfate; IL, interleukin; KO, knockout; Th, T helper cell; TNBS, trinitrobenzene sulfonic acid; VDR, vitamin D receptor.

had more IL-17 in the colon but took longer than vitamin D-sufficient mice to clear the *C. rodentium* infection [127]. Reduced numbers of ILC3 and lower IL-22 were shown to be the cause of the early lethality in the vitamin D-deficient mice [127]. Treatment of mice with 1,25(OH)<sub>2</sub>D increased *C. rodentium* shedding but reduced colitis (Table 97.2; [156]). Vitamin D regulates the immune response and colitis following enteric infection.

Chemical treatment with either trinitrobenzene sulfonic acid (TNBS) or dextran sodium sulfate (DSS) is a way to induce inflammation in the gastrointestinal tract. TNBS and DSS colitis both are induced in mice without T and B cells, and therefore, these models are useful for studying innate immune dysfunction [151]. 1,25(OH)<sub>2</sub>D administration to mice was effective at reducing TNBS-induced colitis (Table 97.2; [141]). Vitamin D-deficient, Cyp27B1 KO, and VDR KO mice were more susceptible to colitis induced with DSS (Table 97.2; [38,147,153]). VDR KO mice had increased mortality following low levels of DSS; as a result of severe intestinal inflammation, loss of barrier function, and endotoxemia (Table 97.2; [106,147]). 1,25(OH)<sub>2</sub>D reduced the severity and improved recovery from DSS and TNBS-induced colitis (Table 97.2; [38,141]). In addition to the immunoregulatory functions of vitamin D, 1,25(OH)<sub>2</sub>D treatments protected mice from chemical injury to the gut, and in part, this was a result of improved barrier function in the presence of 1,25(OH)<sub>2</sub>D.

There is one model of IBD that failed to show beneficial effects of 1,25(OH)<sub>2</sub>D. IL-2 KO mice develop IBD because of an absence of Treg cells [154]. Interestingly the commensal microbiota are not important in the development of colitis in IL-2 KO mice [151]. 1,25(OH)<sub>2</sub>D treatment had no effect on IBD symptoms in the IL-2 KO mice (Table 97.2; [154]). The success of 1,25(OH)<sub>2</sub>D treatment in vivo in two chemical injury models, a T cell transfer model and IL-10 KO mice,

and lack of success in the IL-2 KO model give important insights into the mechanisms underlying the effects of vitamin D as a regulator of intestinal inflammation.

## 5.1 Vitamin D and the microbiota

Over the lifespan factors including early-life events, diet and antibiotic use affects the microbiota and impacts the development of immune-mediated disease. Vitamin D status has been shown to affect the microbiota [160]. Evidence to demonstrate an effect of vitamin D on the microbiota has been shown convincingly in rodents that are on identical diets that differ only in the vitamin D content. Vitamin D-deficient mice have higher Firmicutes and lower numbers of short-chain fatty acid producing bacteria than vitamin D-sufficient mice [67]. Transplantation of the microbiota was able to reproduce the effects of vitamin D to increase the FoxP3<sup>+</sup>/RORγt T regs [67]. Vitamin D-sufficient mice had lower numbers of the Proteobacter phyla and Helicobacter family members than vitamin D-deficient mice (Fig. 97.1; [38]). 1,25(OH)<sub>2</sub>D treatment, of vitamin D-deficient mice, reduced Helicobacter numbers in the mice [38]. Two human trials that supplemented vitamin D in healthy individuals and raised serum 25(OH)D levels were done [161,162]. One trial failed to show significant effects on the microbiota [161,162], while the other showed lower Proteobacteria phyla members with vitamin D intervention [163]. The effects of vitamin D on the microbiota likely depend on the diet, vitamin D status, and other parameters that are difficult to control in human trials. In addition, the effects of vitamin D on the microbiome of a healthy individual may be different than the effect in a patient with dysbiosis. Determining the effectiveness of vitamin D interventions on the microbiota in humans depends on the health and diet of the participants.

Experimentally, the effects of vitamin D on the microbiota have been done using germ-free mice. Germ-free mice have dense bones and fewer osteoclasts than mice with an intact microbiome [164]. The metabolism of vitamin D in germ-free mice was disrupted and mice were hypocalcemic, with very high fibroblast growth factor (FGF)-23, and low serum 25(OH)D/1,25(OH)<sub>2</sub>D levels [165]. Microbial colonization with commensals resulted in mild inflammation followed by inhibition of FGF-23 and then normalization of the serum 25(OH)D levels [165]. Infection of the germ-free mice with a pathogen, reduced serum 25(OH)D levels further, before eventually resolving the infection and reinstating homeostasis and raising serum 25(OH)D levels [165]. In addition, antibiotic disruption of the microbiota increased FGF-23 and disrupted vitamin D metabolism [166]. The microbiota and antibiotics regulate inflammation and FGF-23 levels that control vitamin D metabolism and calcium homeostasis.

Mice and humans with IBD have dysbiosis. The effects of vitamin D to improve experimental colitis are linked to the effects of vitamin D on the microbiota. Vitamin D-deficient and VDR KO mice had microbial dysbiosis that contributed to increased susceptibility to DSS colitis [38]. Antibiotic treatment of the mice eliminated the effect of vitamin D/VDR deficiency on susceptibility to DSS colitis [38]. The inhibition of pathogenic *Helicobacter* bacteria, by either 1,25(OH)<sub>2</sub>D or antibiotics treatment, corresponded with protection from DSS colitis [38]. VDR KO mice or vitamin D-deficient mice had increased barrier dysfunction, dysbiosis of the microbiota, and more intestinal inflammation following infection [155,167,168]. Of note the composition of the commensal flora in vitamin D-deficient or VDR KO mice resembled the dysbiosis noted in IBD patients with fewer commensal from the Firmicutes phyla and higher potentially pathogenic Proteobacteria and Actinobacteria phyla members (Fig. 97.1; [38,107]). High-dose vitamin D treatment reduced Proteobacteria phyla members [163]. In three participants that were *Helicobacter pylori* positive, there was a reduction in *Helicobacter* at the end of the 8 week vitamin D intervention [163]. Clinical trials of vitamin D to manipulate the microbiota were somewhat effective in patients with multiple sclerosis, cystic fibrosis, diabetes, CD, and UC [161,162,169–172]. Vitamin D has been shown to increase Bacteroidetes and decrease Firmicutes [161,162,169–172]. The human and mouse data point to vitamin D regulation of the commensal microbiota that helps to maintain gastrointestinal homeostasis.

## 6. Current treatments for inflammatory bowel disease

There is no cure for IBD. Immunosuppressive drugs are used to induce remission but are not used as

maintenance drugs because of their toxicity and side effects. For maintenance, there are a number of different options ranging from immunomodulators to biological therapies. The use of these newer treatments has resulted in the improved quality of life for patients living with IBD. 5-Aminosalicylic acid (5-ASA) and corticosteroids are the standard first-line therapies for inducing remission in patients with mild to moderate IBD. 5-ASA has been shown to inhibit the synthesis of prostaglandin, leukotrienes, and IL-1 $\beta$  and to suppress the activation of NF- $\kappa$ B by TNF- $\alpha$  and IL-1 [173]. 5-ASA is a broad antiinflammatory agent that has been shown to be effective for treating mild to moderate UC but is less effective for CD [174]. Glucocorticoids, such as prednisone, nonspecifically suppress the immune system and are used to treat moderate to severe CD and UC. Treatment with glucocorticoids is an effective way of inducing remission; however, prolonged use results in steroid dependence or resistance [175]. Although glucocorticoids and 5-ASA are effective treatments to induce remission, not all patients respond. Failure to respond to drug therapy is associated with a worse prognosis, including increased risk of surgery, risk of disability, and an increased risk of infection.

The development of other immunosuppressive drugs has improved treatment for maintaining remission of IBD and has been shown to be useful for limiting long-term use of glucocorticoids [6,176]. Azathioprine, methotrexate, and cyclosporin suppress inflammation by limiting T cell activity. It has been demonstrated that azathioprine treatment is effective for inducing remission in patients with mild IBD and maintaining remission [177]. Methotrexate inhibits the enzymes involved in the nucleoside synthesis and consequently suppresses T cell activation and proliferation and inhibits the expression of adhesion molecules [177]. Treatment with methotrexate is effective in CD but less so for UC [178]. Cyclosporin is a widely used immunosuppressant that inhibits T cell activation by suppressing calcium activation pathway via the inhibition of calcineurin [179]. Cyclosporin has been shown to be effective for treating patients with severe, steroid-refractory UC but has not proven to be effective in patients with severe CD [180].

Within the past 15 years, a number of new biological therapies have been developed and found to be useful to treat IBD. Biologicals target specific aspects of the immune response that contribute to intestinal inflammation. Infliximab (Remicade) and adalimumab (Humira) are some of the first biologics that have been shown to be effective therapy options for patients with IBD [181]. Infliximab and adalimumab are both humanized antibodies that block the activity of TNF- $\alpha$ . These TNF- $\alpha$  blocking drugs were first shown to be effective in patients with arthritis. In 1998, the FDA approved the use of infliximab to treat patients with moderately and severely active IBD who do not respond to conventional

treatment. One of the main complications that exist with the use of this family of drugs is that patients can develop specific immunity against the therapy and stop responding [182]. Since the introduction of the TNF blockers, there have been many other biologicals developed that target other inflammatory cytokines or the cytokine receptors including those for IL-12, IFN- $\gamma$ , and IL-6 [183]. There are now biologicals that antagonize T cells and some that target leukocyte migration [183,184]. These treatments are very expensive, and it is estimated that the use of biologicals to treat IBD results in healthcare costs of more than \$200 billion worldwide [185]. There are many drawbacks to the biologics including expense, required medical personnel to administer, and increased susceptibility to opportunistic infection and cancer. Presently, patients with IBD require lifelong treatment. Because of the expense and risks of all of the current therapies, IBD researchers and patients are interested in exploring alternative strategies to limit intestinal inflammation.

### 6.1 Microbial interventions and IBD

Strategies to modify the microbiota and correct dysbiosis in patients with IBD are of recent interest. One approach would be to use antibiotics to eliminate potential pathogens. Oral antibiotic treatment can be beneficial in models of IBD by eliminating bacteria that are inducing an inappropriate immune response [186]. The use of antibiotics in one study was associated with an increased risk of inflammation, presumably because good microbial flora was eliminated in the gastrointestinal tract [32]. Antibiotic resistance would be an additional barrier to using antibiotics for long-term treatment of IBD. A different approach would involve the transplantation of microbiota from a healthy individual (fecal microbial transplantation [FMT]) to replace the microbiota. FMT has been shown to be effective for the treatment for *Clostridioides difficile* (*C. difficile*) infection [187]. Interestingly, patients with *C. difficile* infection have dysbiosis that resembles vitamin D deficiency; high Proteobacteria and high Firmicutes/Bacteroidetes ratios (Fig. 97.1; [161–163,169–172,188]). Vitamin D-supplemented mothers were less likely to have infants that developed *C. difficile* infections [189]. While FMT for both CD and UC has shown some success, the longevity of the microbial changes or the optimal donor microbes are still not well described [39,40]. Alternatively, probiotics contain viable, defined microorganisms that, when administered in adequate amounts, alter the microflora of the host [190,191]. Probiotics have been shown to have some effectiveness in IBD

patients to induce or maintain remission [191]. Dietary treatments can also shift the microbiota [190]. The production of short-chain fatty acids by the microbial digestion of fiber induces regulatory T cells and suppresses experimental colitis [59,60]. Changes in the microbiota induced through FMT or probiotics may be short lived without additional changes in diet or lifestyle that impact the microbiome. If found to be effective dietary interventions, probiotics hold the promise of treatments that have few side effects and could be used to augment or even replace some of the maintenance therapies for IBD patients.

## 7. Vitamin D as a treatment option for inflammatory bowel disease

Vitamin D and its active metabolites and analogs could be a safe and effective adjunct to the therapies available to treat or prevent IBD. Two small open label trials (one with alfalcidol [192], and one with vitamin D [94]) showed a decrease in CD activity index with vitamin D intervention. Jorgensen showed in a randomized double-blind placebo-controlled study that vitamin D intervention insignificantly ( $P = .0567$ ) reduced the relapse rate in patients with CD [193]. Vitamin D interventions of 2000 IU for 6 months reduced the IBD activity score in children [194]. A recent metaanalysis of 18 randomized controlled trials also indicated that vitamin D interventions improved vitamin D status and prevented relapse [195]. Several issues [196] still need to be resolved for better vitamin D interventions, including the following: (1) What is the appropriate dose and frequency of delivery for the vitamin D intervention? (2) Does it matter what other therapies a patient is on? (3) Can patients with either UC or CD or both benefit? (4) What is the target 25(OH)D levels? (5) Will there be an effect of vitamin D on severe UC or CD? 1,25(OH) $_2$ D or analogs of 1,25(OH) $_2$ D have been shown to be effective treatments of experimental IBD. It is possible that 1,25(OH) $_2$ D rather than vitamin D itself could suppress mild to moderate IBD and perhaps be a glucocorticoid-sparing drug. The benefits of vitamin D in IBD could be multiple, including a reduction in inflammation, alterations in the commensal microbiota and the decreased severity of the disease as well as resolution of secondary hyperparathyroidism and improvement in BMD [192]. However, evaluation of 25(OH)D status has not been established as standard of care in patients with IBD. Even when vitamin D deficiency is detected, there are no established guidelines for treatment in children or adult IBD patients [78].

## 8. Conclusions

Vitamin D is one of several environmental factors that impact the development of UC and CD. Vitamin D-deficient mice develop severe forms of experimental IBD that can be suppressed by treatment with vitamin D/1,25(OH)<sub>2</sub>D. The targets of vitamin D that have been identified in IBD include reductions in pathogenic T cells, induction of regulatory T cells, improved barrier function, induction of pathogen recognition receptors in innate immune cells, and alterations in the microbiota. There is some clinical data that suggests that improving vitamin D status would benefit patients with IBD especially CD. More work needs to be done to understand the mechanisms by which vitamin D maintains gastrointestinal homeostasis and how to best translate the current findings to treating and/or preventing IBD.

## 9. Summary points

- Vitamin D status and the microbiota are two environmental factors that impact the development of IBD.
- Vitamin D or 1,25(OH)<sub>2</sub>D treatments reduced experimental colitis in multiple different animal models.
- Vitamin D insufficiency is associated with increased UC and CD severity.
- Vitamin D regulates innate and acquired immunity to control inflammation in the gastrointestinal tract.
- The beneficial effects of vitamin D in IBD are due in part to the regulation of the gut microbiota.
- Vitamin D interventions might be effective, safe, low-cost therapies that alter the microbiota and improve gastrointestinal health.

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# Vitamin D and tuberculosis

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## OBJECTIVES

- To describe the human immune response to *Mycobacterium tuberculosis*, and how it is modulated by vitamin D.
- To review historical evidence relating to a potential role for vitamin D supplementation in tuberculosis prevention or treatment.
- To summarize evidence from observational epidemiological studies and randomized controlled trials of vitamin D for the prevention or treatment of tuberculosis infection or disease.

## 1. Introduction

Tuberculosis (TB) is caused by infection with the gram-positive acid-fast bacterium *Mycobacterium tuberculosis* (MTB), which was first described by Robert Koch in 1882 [1]. The disease has troubled humans for millennia [2] and is a major global public health problem today. The global prevalence of latent MTB infection (LTBI) has been estimated at 23% [3] and this carries a 5%–20% lifetime risk of reactivation disease in people who are not infected with human immunodeficiency virus (HIV) [4]; reactivation rates higher than 10% per annum have been reported in HIV-infected people [5]. The World Health Organization (WHO) estimates that in 2021 there were 10.6 million incident cases of active TB, and 1.6 million deaths from TB worldwide. This represents a stagnation in the annual decline in TB incidence, and an increase in estimated TB deaths

compared with recent years, both of which have been attributed to adverse impacts of the COVID-19 pandemic on TB control programs [6].

Global efforts to control the TB epidemic have focused on prevention of active disease by vaccination with *Mycobacterium bovis* Bacille Calmette Guérin (BCG) and treatment of LTBI and active TB with combination antimicrobial therapy [7]. The protection imparted by BCG vaccine against TB is highly variable and has been reported to vary from nil to 94%, with greater protection observed at higher latitudes [8]. Treatment of LTBI with isoniazid reduces risk of reactivation disease both in the absence and in the presence of HIV infection [9,10]. Treatment of active TB with conventional short-course chemotherapy (comprising rifampicin, isoniazid, pyrazinamide, and ethambutol for 2 months, followed by rifampicin and isoniazid for 4 months) is effective for the treatment of drug-sensitive disease; however, a significant proportion of patients do not complete treatment [11], resulting in the emergence of drug-resistant strains, which require prolonged therapy with second-line antimicrobial therapy and which cause significant morbidity and mortality [12]. The development of new agents to prevent acquisition or reactivation of LTBI and to allow shortening of antimicrobial therapy regimens for active TB without loss of efficacy is a research priority. This chapter reviews evidence from studies conducted both in vitro and in vivo investigating mechanisms by which vitamin D metabolites modulate immune responses to MTB, and exploring whether vitamin D may have a role in the prevention and treatment of TB. I begin by reviewing in vitro studies that have contributed to the current understanding of the immune response in tuberculosis.

## 2. The immune response in tuberculosis

MTB may enter the human host by inhalation, ingestion, or inoculation; of these routes, inhalation is the most common. Individuals with infectious pulmonary TB expectorate an aerosol of droplet nuclei 1–2  $\mu\text{m}$  in size containing the pathogen, which enter the alveoli where they are phagocytosed by alveolar macrophages, neutrophils, or dendritic cells. Phagocytosis is an active process in which bound mycobacteria are surrounded by phagocyte membrane and then internalized in membrane-enclosed vesicles called phagosomes. Phagosomes are then acidified, and fuse with lysosomes, membrane-enclosed granules containing antimicrobial peptides and hydrolytic enzymes; phagolysosomal fusion is facilitated by the induction of autophagy, a catalytic process involving degradation of cytosolic contents. The importance of this process in host defense against MTB is highlighted by the observation that inhibition of phagosome–lysosome fusion is a key mycobacterial defense strategy [13], which is mediated by the generation of ammonia by virulent mycobacteria [14] and by retention of tryptophan aspartate-containing coat protein (TACO) in the phagosomal membrane [15]. Activation of phagocytic cells also results in induction of innate antimicrobial responses, such as the generation of reactive nitrogen and oxygen intermediates, induction of antimicrobial peptides, production of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-12 (IL-12), and measures to limit availability of lipid droplets to the pathogen [16]. MTB may also be phagocytosed by respiratory epithelial cells, which exert anti-mycobacterial activity via induction of antimicrobial peptides [17] (see Chapters 94 and 95 for more detailed information on this topic).

If innate antimicrobial responses are non-sterilizing, antigen-presenting cells migrate from the lung via the lymph ducts to the lymph node, where presentation of mycobacterial antigens on major histocompatibility complex class II (MHC II) molecules leads to activation and expansion of antigen-specific T cell populations that mediate the adaptive immune response. Activated T cells migrate back to the site of infection in the lung via the bloodstream, where they surround infected macrophages to form granulomata. Activated T cells interact with infected macrophages within the granuloma: CD4<sup>+</sup> T cells secrete cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), which orchestrate the immune response to the pathogen and enhance macrophage activation, while CD8<sup>+</sup> T cells may kill infected macrophages [18] (see Chapters 94–96). The critical importance of TNF, IL-12, and IFN- $\gamma$  in the anti-mycobacterial immune response is illustrated by clinical observations that individuals with defects in these

pathways have heightened susceptibility to mycobacterial infection [19–21]. However, active tuberculosis is associated with expression of type I interferons [22], which may suppress IFN- $\gamma$ -inducible anti-mycobacterial responses [23].

The interactions between host and pathogen described before have several potential outcomes. It is well recognized that a significant proportion of individuals exposed to infectious cases of TB do not subsequently develop evidence of an adaptive response to MTB [24], suggesting that innate mechanisms may be effective in eliminating infection before initiation of the adaptive immune response [25]. A second group of individuals may develop evidence of an early adaptive response (i.e., MTB antigen-stimulated T cell production of IFN- $\gamma$ ), which subsequently reverts from positive to negative [26]: this may signify successful elimination of infection following initiation of an adaptive response. A third group may develop persistent evidence of T cell priming without developing clinical or radiological features of active disease; these individuals are thought to have LTBI, with MTB held in a state of non-replicating persistence. Individuals with LTBI may remain asymptomatic for life or may subsequently progress to develop active disease (secondary TB) [27]. Finally, in some individuals, the immune response does not restrict growth of the organism, and active disease develops shortly after infection (primary TB) [28].

## 3. Influence of vitamin D on the immune response to MTB

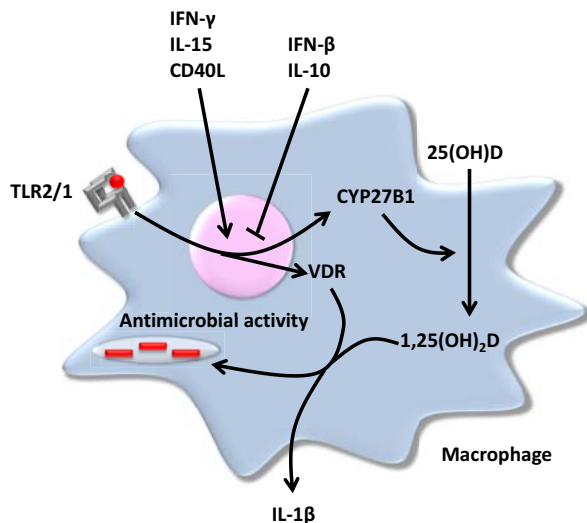
Humans acquire vitamin D via cutaneous synthesis as a result of the action of solar ultraviolet B radiation on 7-dehydrocholesterol in the skin, or from the diet, principally by consumption of oily fish or dietary supplements (see Chapter 3 and Chapters 55–58). Vitamin D from either source is metabolized by the liver to form 25-hydroxyvitamin D (25(OH)D), the major circulating metabolite of vitamin D whose serum concentration is used to define vitamin D status. 25(OH)D circulates bound to vitamin D-binding protein (DBP) and is metabolized by the enzyme 25(OH)D-1 $\alpha$ -hydroxylase (CYP27B1), expressed in the kidney, as well as in leukocytes, gastrointestinal tract, skin, vascular endothelium, and placenta to form 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), the steroid hormone, and active metabolite of vitamin D. 1,25(OH)<sub>2</sub>D induces biological actions by ligating vitamin D receptor (VDR). Both 1,25(OH)<sub>2</sub>D and 25(OH)D are catabolized by the 24-hydroxylase enzyme CYP24A1 to form the relatively biologically inactive metabolites 1,24,25-trihydroxyvitamin D and 24,25-dihydroxyvitamin D, respectively.

With the exception of two reports [29,30], vitamin D and its metabolites have not been shown to possess anti-mycobacterial activity in the absence of cells. However, 1,25(OH)<sub>2</sub>D has long been recognized to induce anti-mycobacterial activity in vitro in mononuclear phagocytes [31]. Its precursor 25(OH)D has also been shown to support induction of innate anti-mycobacterial responses [32], as illustrated in Fig. 98.1. Ligation of macrophage Toll-like receptor (TLR) 2/1 heterodimers by mycobacterial lipoprotein induces expression of VDR and CYP27B1 [33,34]. CYP27B1 is also induced by IL-15 [34] (produced by peripheral blood monocytes in response to TLR2/1 stimulation), CD40 ligand [35], and IFN- $\gamma$  [36] (produced by CD4 + T helper type 1 [Th1] cells): indeed, human macrophages require 25(OH)D for induction of IFN- $\gamma$ -mediated anti-mycobacterial activity [32]. Induction of CYP27B1 and VDR by IFN- $\gamma$  is blocked by IFN- $\beta$  and IL-10 [23]. Because extra-renal CYP27B1 follows first order kinetics, the rate at which it synthesizes 1,25(OH)<sub>2</sub>D depends on availability of 25(OH)D substrate [37] (see Chapter 9). Orally ingested vitamin D is freely converted to 25(OH)D [38], and this provides the rationale for administering “parent” vitamin D to induce anti-mycobacterial responses at the site of infection.

1,25(OH)<sub>2</sub>D modulates immune responses by ligating membrane VDR to induce rapid effects (within minutes), or nuclear VDR to induce genomic effects (within

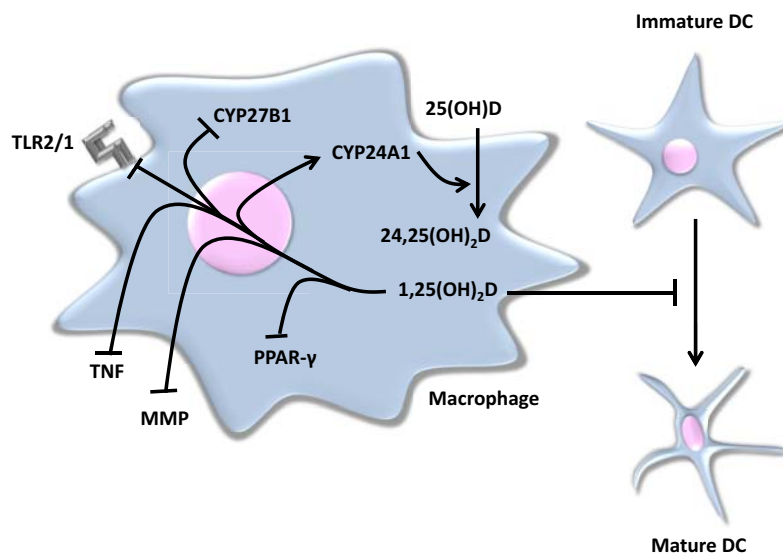
hours) [39]. Experiments using selective agonists and antagonists of these two receptors indicate that ligation of nuclear VDR is both necessary and sufficient for induction of anti-mycobacterial responses by 1,25(OH)<sub>2</sub>D in vitro [40]. 1,25(OH)<sub>2</sub>D modulates the host response to mycobacterial infection by pleiotropic mechanisms including the induction of reactive nitrogen and oxygen intermediates [41,42], down-regulation of TACO [43], promotion of phagolysosome fusion [44], and induction of antimicrobial peptides including cathelicidin (LL-37) [33,40] and (in synergy with IL-1 $\beta$ ) human beta defensin 2 [45]. Cathelicidin LL-37 possesses anti-mycobacterial activity [25,33] and induces autophagy [46,47], which inhibits replication of both MTB and HIV in co-infected macrophages [48]. 1,25(OH)<sub>2</sub>D-induced anti-mycobacterial activity has been reported to be dependent on expression of *hCAP18*, the gene encoding the parent molecule from which LL-37 is cleaved [49], and potentiated by co-culture with the aromatic fatty acid phenylbutyrate [50,51]. 1,25(OH)<sub>2</sub>D has also been reported to enhance expression and secretion of IL-1 $\beta$  by MTB-infected macrophages, which induces anti-mycobacterial activity in respiratory epithelial cells via induction of human  $\beta$ -defensin 2 [17].

In addition to inducing innate antimicrobial responses, 1,25(OH)<sub>2</sub>D modulates macrophage, dendritic cell, and T cell function to regulate innate and adaptive immune responses to infection. In the macrophage, 1,25(OH)<sub>2</sub>D directly regulates its own synthesis and catabolism via inhibition of *CYP27B1* and induction of *CYP24A1*, respectively; it also downregulates expression of TLR2/1 [52], TNF [40], and matrix metalloproteinase (MMP) enzymes that are implicated in the pathogenesis of pulmonary cavitation [53], as well as downregulating PPAR- $\gamma$  to abrogate infection-induced accumulation of lipid droplets required for intracellular growth of MTB [54] (Fig. 98.2). 1,25(OH)<sub>2</sub>D also inhibits dendritic cell maturation [55], downregulates MHC class II expression, inhibits secretion of IL-12, and induces secretion of IL-10 [40]; together, these actions skew development of null T helper cells away from a Th1 phenotype and toward T helper type 2 (Th2) and T regulatory cell profiles [55–57] (Fig. 98.3). Finally, in the granuloma, 1,25(OH)<sub>2</sub>D attenuates antigen presentation via inhibition of macrophage MHC class II expression and directly modulates T helper cell cytokine secretion, stimulating production of IL-4 from Th2 cells and inhibiting IFN- $\gamma$  production from Th1 cells. Since IL-4 induces catabolism of 25(OH)D in a CYP24A1-dependent manner [58], and IFN- $\gamma$  induces *CYP27B1* expression [36], this represents another negative feedback loop via which 1,25(OH)<sub>2</sub>D regulates its own concentration at the site of mycobacterial infection (Fig. 98.4). Further discussion of the effects of vitamin D on immune responses to bacterial infection can be found in Chapter 94.

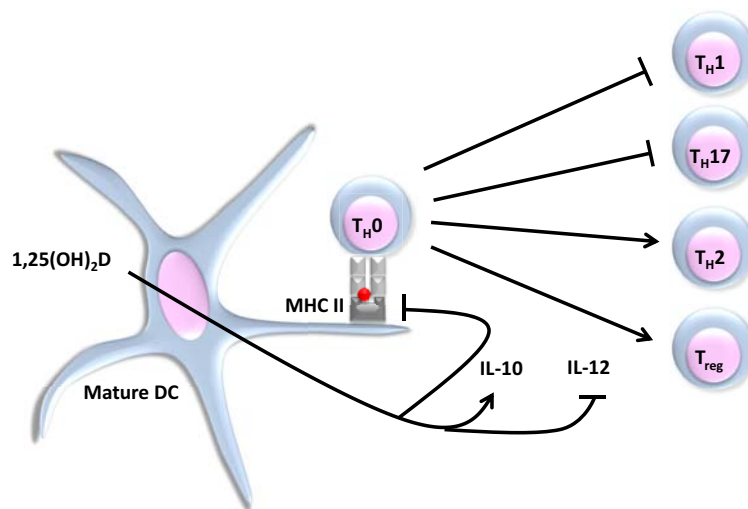


**FIGURE 98.1** 25-hydroxyvitamin D supports macrophage TLR ligand-induced innate antimicrobial responses. Mycobacterial lipoproteins ligate TLR2/1 heterodimers on the macrophage surface, leading to induction of *CYP27B1* and VDR. IL-15, CD40L and IFN- $\gamma$  also induce *CYP27B1*, but this is blocked by IFN- $\beta$  and IL-10. In the presence of adequate 25(OH)D substrate, *CYP27B1* synthesizes 1,25(OH)<sub>2</sub>D, which ligates VDR to initiate pleiotropic antimicrobial responses (including induction of reactive nitrogen and oxygen intermediates, antimicrobial peptides, and autophagy) and secretion of IL-1 $\beta$ .





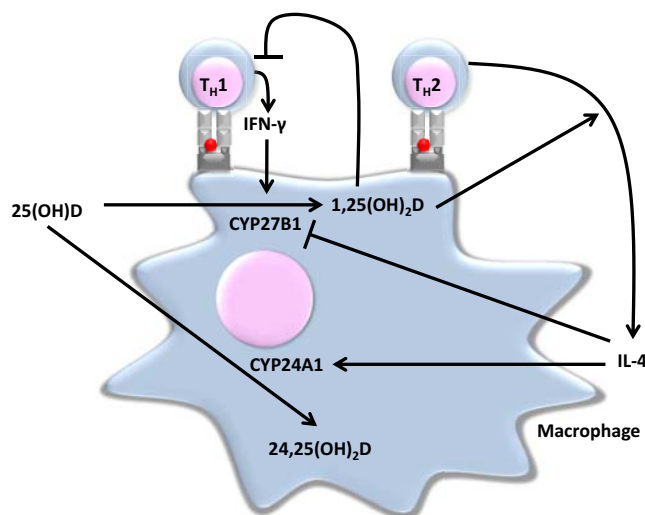
**FIGURE 98.2** 1,25(OH)<sub>2</sub>D regulates innate inflammatory responses to mycobacterial infection. 1,25(OH)<sub>2</sub>D downregulates expression of TLR2/1, suppresses secretion of TNF and MMP enzymes implicated in the pathogenesis of pulmonary cavitation, and inhibits dendritic cell maturation. It also downregulates PPAR-γ to abrogate infection-induced accumulation of lipid droplets required for intracellular growth of *Mycobacterium tuberculosis*. 1,25(OH)<sub>2</sub>D directly regulates its own synthesis and catabolism via inhibition of CYP27B1 and induction of CYP24A1, respectively. Arrows represent stimulatory actions, and bars represent inhibitory actions.



**FIGURE 98.3** 1,25(OH)<sub>2</sub>D influences T helper cell profile. In secondary lymphoid tissue, 1,25(OH)<sub>2</sub>D acts on the dendritic cell to enhance secretion of IL-10 while suppressing secretion of IL-12 and expression of MHC class II. Together, these actions favor development of null T helper cells toward Th2 and T regulatory phenotypes, and away from Th1 and Th17 phenotypes. Arrows represent stimulatory actions, and bars represent inhibitory actions.

Given the suppressive effects of 1,25(OH)<sub>2</sub>D on Th1 responses reviewed before, and the clinical observation that such responses are required for host defense against intra-cellular pathogens, there is clearly potential for in vivo vitamin D supplementation to attenuate anti-mycobacterial responses as well as to enhance them. However, while abrogation of the IL-12 and IFN-γ pathways clearly results in exquisite susceptibility to

mycobacterial infection [20,21], it does not necessarily follow that partial attenuation of these responses will lead to impaired protection against, or resolution of, TB. In active disease, in particular, Th1 cytokines may cause immunopathology [59]; an immunomodulatory agent with the ability to induce antimicrobial activity and inhibit Th1-induced immunopathology simultaneously might therefore have potential as an adjunct to



**FIGURE 98.4** 1,25(OH)<sub>2</sub>D modulates adaptive responses to mycobacterial infection in the granuloma. 1,25(OH)<sub>2</sub>D stimulates secretion of IL-4 from Th2 cells and inhibits secretion of IFN-γ from Th1 cells to regulate its concentration at the site of mycobacterial infection. Arrows represent stimulatory actions, and bars represent inhibitory actions.

anti-tuberculous therapy, for example. Only clinical studies can resolve the question of whether stimulatory or suppressive immunomodulatory actions of vitamin D are dominant in vivo. A review of such studies follows, beginning with a consideration of the historical literature.

#### 4. Historical studies

The clinical features of vitamin D deficiency were first described in 1651, when Glisson, Bate, and Regemorter published “A treatise of the rickets: being a disease common to children” [60]. In addition to noting the classical musculoskeletal features of rickets, the authors made the following observation from an autopsy of an infant with the condition: “One amongst us doth attest, that he saw glandulous knobs and bunches so numerous that they seemed to equalise, if not exceed, the magnitude of the lungs themselves; they were situated between the lungs and the mediastinum ... and were extended from the Canel bone to the Diaphragma.” TB is a well-recognized cause of mediastinal lymphadenopathy in children [61], and it is interesting to speculate whether this represents the earliest case report of TB associated with vitamin D deficiency.

Some 200 years later, physicians at the Hospital for Consumption and Diseases of the Chest, Brompton, London, conducted an intervention study to evaluate the effects of administering cod liver oil (which contains high concentrations of both vitamin D and vitamin A) to

patients with “pulmonary consumption.” A total of 542 patients were treated with thrice daily doses of cod liver oil ranging from 3.6 to 42 mL, and their clinical outcomes were compared with those of a control group of 542 patients who did not receive the oil: 19% of patients receiving cod liver oil died, compared with 33% of those who did not receive it. It was concluded that “Cod Liver Oil possesses the property of controlling Pulmonary Consumption to a greater extent than any other agent hitherto tried” [62]. This report represents some of the earliest evidence that administration of a preparation containing vitamin D improved clinical outcome in patients with TB, although it should be noted that allocation was not randomized, bacteriological confirmation of diagnosis was lacking (the study predated Koch’s discovery of MTB by more than 3 decades), and that any beneficial effects of cod liver oil may be attributable to its content of vitamin A rather than vitamin D [63]. The first TB sanatorium was opened in Gorborsdorf, Germany (today Sokolowsko, Poland) in 1859, and heliotherapy (exposure of TB patients to the sun, thereby promoting cutaneous synthesis of vitamin D) subsequently became common practice and was credited with improvements in clinical outcome in many cases [64]. In 1903, Niels Finsen was awarded the Nobel Prize in Physiology or Medicine for his discovery that short-wave ultraviolet light was effective in the treatment of cutaneous TB [65]. While it is possible that these effects were mediated by induction of cutaneous vitamin D synthesis, it should be noted that ultraviolet irradiation modulates immune responses via vitamin D-independent pathways [66] and that it also directly inactivates MTB [67].

In 1914, McCollum and coworkers subsequently conducted a series of experiments, leading to the discovery of vitamin D. They isolated a substance from butterfat, necessary for prevention of xerophthalmia in rats, and named it “fat-soluble factor” A [68]. They subsequently reported that heated oxidized cod liver oil could not prevent xerophthalmia but could cure rickets in rats and concluded that “fat-soluble factor A” consisted of two entities, one that could prevent xerophthalmia (subsequently called vitamin A), and one that cured rickets (subsequently called vitamin D, as the terms vitamin B and vitamin C had already been coined) [69]. Vitamin D<sub>2</sub> was purified and crystallized in 1931 [70], and Charpy subsequently pioneered the use of pharmacologic doses ( $\geq 1.25$  mg or 50,000 IU daily) of vitamin D<sub>2</sub> to treat cutaneous TB [71]. Vitamin D<sub>2</sub> was also used to treat pulmonary TB, both as a single agent and, following the introduction of effective anti-tuberculous chemotherapy, as an adjunct to antibiotic treatment. Case series reporting this practice are summarized in Table 98.1: broadly, those in which doses of vitamin D  $\geq 1.25$  mg/day ( $\geq 50,000$  IU/day) had been

**TABLE 98.1** Case series investigating effects of administering vitamin D to patients with tuberculosis.

References	Participants setting	Dose of vitamin D	Concurrent antimicrobial treatment	Therapeutic response	Adverse events
Brincourt et al., [72]	" > 100" patients with PTB, France	600,000 IU D2 every 10 days for up to 4 months (maximum dose 7.2M IU)	Antibiotic treatment—not specified	Rapid liquefaction of caseating necrosis; decreased fibrotic sequelae	One patient developed hypercalcemia after 7.2M IU total dose; nephrocalcinosis did not ensue
Trautwein et al., [73]	35 patients with PTB and cutaneous TB, Germany	200,000 IU D2 3 × / week	PAS or TB.I	19/35 made "good/very good response," 10/35 "some/temporary" response	4/35 developed hypercalcemia (> 3 mmol/L)
Fielding et al., [74]	7 adult patients with PTB, United Kingdom	600,000 IU D2 IM weekly for 3 doses, fortnightly for 4 weeks, monthly for 4 months (total dose 5.4M IU)	Streptomycin and PAS	"Cavitation reduced" in 6/7; 1/7 (with streptomycin-resistant disease) deteriorated	No toxic effects attributed to calciferol; anorexia and vomiting "in some cases" attributed to PAS
Gerecke et al., [75]	81 pts with PTB, Germany	40,000 IU daily	With or without TB.I	Four cases with combination showed response; none taking vitamin D alone showed a response	Not reported
Sude et al., [76]	9 patients with PTB, Germany	600,000 IU D2 3 × / week for 1st week, 2 × /week in 2nd to 4th weeks, then weekly	No	None improved; 5 deteriorated	2 patients developed hypertension; 1 patient discontinued vitamin D due to symptomatic hypercalcemia
Jongmans et al., [77]	123 patients with PTB, Holland	90,000 IU D2 po daily for up to 12 months	No	Not reported	5 patients experienced exacerbation of symptoms
Ianovskaya et al., [78]	78 patients with PTB and cutaneous TB, Russia	50,000 to 100,000 IU D2 po daily	No	42/78 improved; 28/78 unchanged; 8/78 deteriorated	16 of the 42 patients who improved experienced exacerbation of symptoms
Seeber et al., [79]	28 patients with PTB	20,000IU D2 daily, increasing to 40,000 IU daily; total dose 6M IU	No	12/28 improved; 13/28 unchanged; 3/28 deteriorated	Not reported
Winterberg et al., [80]	52 patients with PTB, Germany	400,000 IU D2 every 5–10 days	Not reported	44/52 improved	Not reported
Feeny et al., [81] English.	21 patients with PTB, United Kingdom	25,000 IU D2 daily po, increasing to 100,000 IU/day	3 mg vitamin B1 daily po	ESR up in 12/21, down in 5/21. 16/21 sputum positive before and after treatment; 2 converted positive to negative; 2 remained negative; 1 produced no sputum	Headache in 7/21; nausea in 16/21; vomiting in 4/21

D2, vitamin D<sub>2</sub>; EPTB, extrapulmonary TB; ESR, erythrocyte sedimentation rate; IU, International Unit of vitamin D (1 IU = 0.025 µg vitamin D); PAS, para-aminosalicylic acid; po, per os; PTB, pulmonary TB.

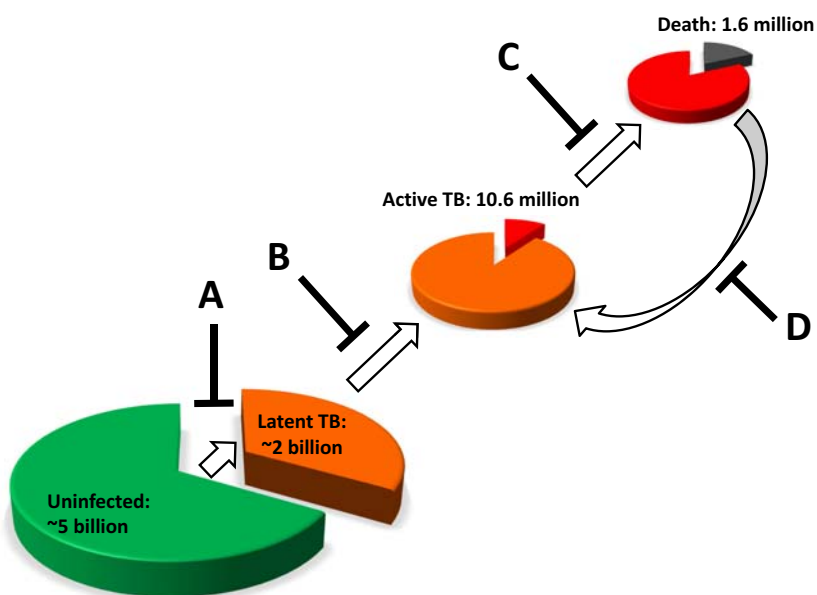
administered reported beneficial effects (despite the lack of control groups), and occasional hypercalcemia was observed. Hypercalcemia had been recognized to associate with TB since 1931, in both the presence and absence of vitamin D supplementation [82]. Indeed, a case report of hypercalcemia arising in a TB patient with end-stage renal disease, associated with elevated circulating concentrations of  $1,25(\text{OH})_2\text{D}$ , led to the discovery of dysregulated CYP27B1 activity in patients with TB—one of the first examples of extra-renal vitamin D metabolism to be recognized beyond sarcoidosis [83].

Taken together, evidence from the in vitro literature and historical studies suggests four potential clinical applications of vitamin D in TB control: prevention of MTB infection, prevention of LTBI reactivation, enhancement of response to antimicrobial therapy, and prevention of recurrent disease (Fig. 98.5). I will now review clinical studies from the modern era, classified according to the outcome measure or endpoint studied, namely, acquisition of MTB infection, incident active tuberculosis (disease), and response to antimicrobial therapy for active disease (treatment studies). In each case, observational and intervention studies will be reviewed in turn.

## 5. Studies with MTB infection as an end point

### 5.1 Observational studies

Cross-sectional and case–control studies conducted in Australia [84], Italy [85], Gambia [86], Mongolia [87,88], and the United States [89] have all reported independent associations between lower circulating  $25(\text{OH})\text{D}$  concentrations and increased risk of MTB infection, although a study in Brazil found no such association [90]. Three cohort studies in Spain both found an association between low serum  $25(\text{OH})\text{D}$  concentrations and increased risk of tuberculin skin test conversion following exposure to a case of infectious TB [91–93], while a population-based longitudinal study in South Africa found an association between vitamin D deficiency in early infancy and increased risk of tuberculin skin test conversion by the age of 2 years [94]. Another cohort study, conducted in Peru, has reported a temporal association between vitamin D deficiency and incident MTB infection in a cohort of TB contacts: a midwinter peak in vitamin D deficiency was followed 6 weeks later by a late-winter peak in tuberculin skin test positivity and 12 weeks after that by an early-summer peak in QuantiFERON positivity [95].



**FIGURE 98.5** Potential roles for vitamin D in the prevention and treatment of tuberculosis. Enhancement of the immune response to *Mycobacterium tuberculosis* by means of vitamin D supplementation might prevent acquisition of latent TB infection in exposed individuals (A) Prevent reactivation of latent TB infection to reduce incidence of active disease (B) Enhance response to antibiotic therapy in active disease, to improve outcome or to allow shortening of antibiotic treatment regimens (C) Or prevent recurrent disease in those who have recovered from a previous episode (D) Arrows represent disease progression, and bars represent potential inhibitory/therapeutic actions of vitamin D. Estimated numbers from the WHO Global Tuberculosis Report, 2022.



## 5.2 Intervention studies

Building on positive findings from a proof-of-concept study showing that vitamin D supplementation augmented anti-mycobacterial immunity in whole blood [96], three randomized controlled trials (RCTs) investigating the influence of vitamin D supplementation on resistance to MTB infection have been conducted to date [97–99]. Ganmaa and colleagues first conducted a pilot study, in which they randomized 120 Mongolian schoolchildren aged 12–15 years to receive a daily oral dose of 800 IU vitamin D<sub>3</sub> or placebo for a period of 6 months. Tuberculin skin tests were performed at baseline and at the end of the trial: these converted from negative to positive in 11% of children receiving vitamin D vs. 27% of those receiving placebo (RR: 0.41; 95% CI: 0.16, 1.09;  $P = .06$ ). These positive results led to conduct a phase 3 RCT of vitamin D for the prevention of MTB infection in the same setting, in which 8851 QuantiFERON-negative schoolchildren aged 6–13 years were randomized to receive a weekly oral dose of placebo or 14,000 IU vitamin D<sub>3</sub> for 3 years [98]. Vitamin D deficiency was very common among study participants at baseline, and the intervention was highly effective in elevating serum 25(OH)D concentrations of participants randomized to the intervention arm. However, no inter-arm differences in risk of QuantiFERON conversion were seen. Another phase 3 RCT investigating effects of weekly vitamin D supplements on risk of QuantiFERON conversion in Cape Town schoolchildren (“ViDiKids,” clinicaltrials.gov identifier NCT02880982) [99] has recently completed and has also yielded null results.

## 6. Studies with active TB disease as end point

### 6.1 Observational studies

In 1985, Davies observed that people migrating to the United Kingdom from countries with a high incidence of LTBI experienced rates of active TB that exceeded rates in their countries of origin and that this increased risk coincided with the development of vitamin D deficiency, probably arising as a result of decreased sun exposure [100]. He suggested that vitamin D deficiency may predispose to reactivation of LTBI, a hypothesis supported by other ecological studies reporting a spring/summer peak in TB notification rates in a wide range of settings, following shortly after the seasonal nadir in population vitamin D status [101,102]. Another ecological study has shown that regional solar radiation is inversely correlated with incidence and severity of tuberculosis in this setting [103], while a cross-sectional investigation conducted in an ethnically diverse group of 462 TB patients in the United Kingdom has reported an

independent association between vitamin D deficiency and risk of extra-pulmonary tuberculosis [104]. This finding raises the possibility that vitamin D deficiency may be implicated in the pathogenesis of extra-pulmonary dissemination, as well as contributing to susceptibility to disease per se.

Further evidence comes from case–control studies investigating whether vitamin D deficiency associates with susceptibility to active TB. More than 50 such primary studies have been conducted to date, with data from them contributing to four meta-analyses [105–108], all of which report statistically significant associations between lower vitamin D status and increased risk of active TB. Potential explanations for an association between vitamin D deficiency and active TB include both causality (i.e., vitamin D deficiency impairs host immune response to MTB and causes susceptibility and extra-pulmonary dissemination), confounding and reverse causality (i.e., active TB causes vitamin D deficiency, due to anorexia, decreased exposure to sunlight in debilitated patients, or MTB-induced dysregulation of vitamin D metabolism [109]).

One approach to eliminate the potential influence of reverse causality using a case–control study design is to investigate whether genetic variation in pathways of vitamin D metabolism, transport, or signaling associates with altered susceptibility to active TB. To date, this line of enquiry has primarily focused on polymorphisms in the VDR. Human VDR is encoded by the *VDR* gene located on chromosome 12q. This gene is polymorphic, and numerous single-nucleotide polymorphisms have been described. The hypothesis that *VDR* variants might associate with susceptibility to active TB was first investigated by Bellamy and colleagues, who reported an association between carriage of the *T* allele of the *TaqI* *VDR* polymorphism and susceptibility to active TB in a case–control study conducted in Gambian adults [110]. Wilkinson and colleagues subsequently reported that associations between susceptibility to TB and carriage of the *T* allele of the *TaqI* *VDR* polymorphism and the *ff* genotype of the *FokI* *VDR* polymorphism in Gujarati Asians living in London were restricted to vitamin D–deficient individuals [111]; this study is the first to report that gene: environment interactions may operate to influence susceptibility to active TB. More than 50 case–control studies investigating the association between *VDR* variants and susceptibility to active TB have now been published. Meta-analyses of these studies report a consistent statistically significant association between the *ff* genotype of the *FokI* polymorphism and susceptibility to active TB in study populations overall (pooled odds ratios ranging from 1.23 to 1.60) [112–115]. Consistent associations for the *TaqI*, *ApaI*, and *BsmI* polymorphisms have not been found overall, although subgroup analysis has revealed an association (odds ratio 1.43)

between the *tt* genotype of the *TaqI* polymorphism and susceptibility to TB in Asian populations [116].

The mechanisms by which *VDR* polymorphisms influence susceptibility to TB are incompletely understood. *FokI* is a nonsynonymous SNP in the translation start site of exon 2 of the *VDR* gene. The SNP is characterized by a C-to-T substitution in a 5' ATG start codon, resulting in a *VDR* protein, which is of 427 amino acids, instead of 424 amino acids, in length [117]. Human monocytes and dendritic cells homozygous for the *F* allele exhibit higher expression of interleukin-12 (IL-12) messenger RNA (mRNA) and protein, and lymphocytes of individuals with the *FF* genotype proliferate more strongly in response to phytohemagglutinin than those of individuals with the *ff* genotype [118]. In contrast to *FokI*, *TaqI* and *BsmI* are synonymous SNPs located in intron 8 and exon 9 of the *VDR* gene, respectively; they are in linkage disequilibrium with each other and with a poly(A) length polymorphism in the 3' untranslated region of the *VDR* gene [119]. Carriage of the *t* allele of the *TaqI* polymorphism has been reported to associate with an increase in *VDR* gene expression. Because the 3'-UTR is a major regulator of mRNA half-life [120], this association was originally thought to be mediated via an effect on mRNA stability [121]; however, subsequent studies have refuted this hypothesis [122,123], and the mechanism for the association is not currently understood.

Further case-control studies have investigated associations between polymorphisms in *Gc* (the gene encoding DBP) and susceptibility to active TB. DBP is a highly expressed multifunctional 58 kDa serum glycoprotein encoded on chromosome 4. Two common polymorphisms at codons 416 and 420 of exon 11 of the *Gc* gene give rise to the three major electrophoretic variants of DBP, termed group-specific component 1 fast (Gc1F), Gc1 slow (Gc1S), and Gc2. These variants differ in their functional characteristics: the Gc1F and Gc1S variants have been reported to have greater affinity for 25(OH)D than the Gc2 variant [124], potentially leading to more efficient delivery of 25(OH)D to the target tissues, while the Gc2 variant is associated with decreased circulating concentrations of 25(OH)D, 1,25(OH)<sub>2</sub>D, and DBP [125,126]. A case-control study conducted in Taiwan has reported an association between the Gc1F allele and susceptibility to TB [127], although studies conducted in India, Russia, Kuwait, and Pakistan did not find any such association [128–131]. A fifth study reported an association between the Gc2 allele of vitamin D-binding protein and susceptibility to active TB among Gujarati Asians living in London. This association was preserved if serum 25(OH)D concentration was < 20 nmol/L, but not if serum 25(OH)D was ≥ 20 nmol/L, suggesting that profound vitamin D deficiency and Gc2 genotype may interact to increase susceptibility to TB [132].

In contrast to the numbers of published cross-sectional and case-control studies, relatively few cohort studies investigating associations between vitamin D status and risk of incident active TB have been conducted. A recent meta-analysis of individual participant data from these studies reported a dose-dependent association between low baseline vitamin D status at baseline and increased risk of incident TB disease subsequently, with risk of TB disease being highest among HIV-positive individuals and in those with severe vitamin D deficiency at baseline [133]. A consistent association was reported by the only other longitudinal study of this kind that has reported since this meta-analysis was conducted [134]. However, confounding cannot be ruled out as an explanation for associations reported in observational studies, and RCTs are needed to establish causality.

## 6.2 Intervention studies

Three phase 3 RCTs investigating potential effects of vitamin D supplementation on risk of incident active TB have reported to date. In the TOV-4 trial, Sudfeld and colleagues randomized 4000 HIV-infected adults in Tanzania with baseline serum 25(OH)D concentrations of less than 75 nmol/L to receive either four weekly oral bolus doses of 50,000 IU vitamin D<sub>3</sub> over 1 month, followed by a daily dose of 2000 IU vitamin D<sub>3</sub> for 11 months, vs. a matching weekly and daily placebo regimen. No difference in risk of incident active TB was seen between arms (hazard ratio 0.78, 95% CI 0.54–1.13). In another trial [98], Ganmaa and colleagues randomized 8851 schoolchildren in Mongolia to receive a weekly oral dose of 14,000 IU vitamin D<sub>3</sub> vs. placebo for 3 years. Again, no effect of the intervention on risk of incident active TB was seen (adjusted risk ratio 0.87, 95% CI 0.49–1.55). Most recently, the ViDiKids trial, conducted in South African schoolchildren, reported no effect of a weekly oral dose of 10,000 IU vitamin D<sub>3</sub> vs. placebo on risk of active TB, although the number of incident cases was small [99].

## 7. Studies of treatment outcome

### 7.1 Observational studies

A handful of observational studies have investigated the relationship between baseline vitamin D status and outcome of anti-tuberculous therapy in patients with active TB. Three cohort studies have reported independent associations between lower vitamin D status and impaired clearance of MTB from the sputum of patients with pulmonary TB during intensive-phase treatment—two in drug-sensitive disease [135,136] and one in

multidrug-resistant (MDR) disease [137]. Additionally, a cohort study conducted in Tanzania has also reported an independent association between vitamin D insufficiency ( $25[\text{OH}]\text{D} < 75 \text{ nmol/L}$ ) and risk of relapse of active tuberculosis in a cohort of 677 patients completing treatment for active TB, 344 of whom had HIV infection [138].

Studies investigating the influence of genetic variation in the vitamin D pathway on treatment outcome are all restricted to the intensive phase of therapy: Roth and colleagues have reported that the *FF* genotype of the *FokI* *VDR* polymorphism and the *Tt* genotype of the *TaqI* *VDR* polymorphism associate with faster sputum culture conversion in a cohort of pulmonary TB patients in Peru [139], while Babb and colleagues reported no difference in time to sputum culture conversion according to *TaqI* or *FokI* *VDR* genotype among South African TB patients [140]. However, Junaid and colleagues did not find any association between sputum smear conversion and polymorphisms in *VDR*, *CYP2R1*, or *DBP* [136].

## 7.2 Intervention studies

In contrast to trials for the prevention of incident active tuberculosis, intervention studies to determine whether adjunctive vitamin D enhances response to antimicrobial therapy can be powered to detect clinically significant effects of treatment with more modest numbers of participants and shorter follow-up. Twenty-one such studies have been published to date, contributing data to six meta-analyses [141–146], all of which have shown no overall effect of adjunctive vitamin D on time to sputum culture conversion. Subgroup analyses in two meta-analyses [144,145] have, however, suggested a possible benefit among patients with MDR TB, which should be investigated in new primary RCTs conducted exclusively in patients with MDR TB.

## 8. Conclusions

A very large body of evidence relating to potential effects of vitamin D in human tuberculosis now exists. Findings from laboratory work have tended to suggest a positive influence of vitamin D metabolites on host response to MTB in cell culture, and observational epidemiological studies have reported associations between higher vitamin D status and favorable clinical outcomes, including lower risk of MTB infection and disease, and better response to antimicrobial therapy in patients with pulmonary disease. By contrast, RCTs of vitamin D for prevention of MTB infection and disease, and for treatment of pulmonary TB, have tended

to yield null results—with the possible exception of patients being treated for MDR TB, where subgroup analyses in some meta-analyses have suggested potential benefit. How can this “disconnect” between findings clinical trials vs. laboratory studies and observational epidemiology be explained?

Broadly, there are two possibilities. It may be that positive results from laboratory studies do not reflect physiological conditions, and positive associations from observational epidemiology are non-causal. Alternatively, RCTs may have yielded false-negative results due to problems with their design or execution.

With regard to laboratory studies, lack of relevance to in vivo responses may reflect investigation of concentrations of  $1,25(\text{OH})_2\text{D}$  that are several orders of magnitude higher than those measured in the circulation or in extracellular fluid in humans [40]. Moreover, cell culture systems may not contain plasma proteins such as DBP or albumin, which may affect bioavailability of vitamin D metabolites in vivo [147]. Additionally, many cell culture systems investigate effects of vitamin D metabolites in a single cell type (most commonly mononuclear phagocytes) [31,42]. This reductionist approach ignores potential effects of vitamin D on the function of other cellular players, including T cells, which may antagonize or regulate effects of vitamin D on macrophage function [40].

With regard to epidemiological studies, non-casual associations may arise via two main mechanisms. First, confounding may be operating, whereby “third factors” related to both exposures and outcomes of interest are either imperfectly measured (residual confounding) or not controlled for at all (unmeasured confounding) in multivariable analyses testing for associations [148]. Alternatively, or additionally, reverse causality may operate, such that outcomes may actually influence the exposures: for example, active TB disease might reduce circulating  $25(\text{OH})\text{D}$  concentrations, either by metabolizing cholecalciferol itself [149] or by dysregulating host vitamin D metabolism [150]—a possibility that has also been raised in other inflammatory pulmonary diseases [151].

With regard to clinical trials, false-negative results may arise as a consequence of factors including participant characteristics (e.g., where baseline vitamin D status is adequate prior to supplementation [152]), use of inappropriate dosing regimens (e.g., if vitamin D is administered at too low a dose to produce sustained elevations in circulating  $25[\text{OH}]\text{D}$  concentrations [153]), masking of vitamin D effects by more effective antimicrobial therapy [154,155], poor adherence to study supplements, high rates of loss to follow-up, or other issues constraining statistical power.

Given the challenges in interpreting results of the aforementioned studies, it may be that Mendelian



randomization studies [156] can yield additional insights into a potential role for vitamin D deficiency in influencing susceptibility to, or severity of, tuberculosis—as they have done for multiple sclerosis [157]. Ultimately, however, consistent null results from RCTs of vitamin D for prevention and treatment of TB hold greater weight with clinical and public health policy-makers than positive results from laboratory and observational studies. Accordingly, international guidelines for the management and control of TB do not specify a role for vitamin D either in the prevention or in the treatment of this disease [158].

## 9. Summary points

- Cod liver oil and vitamin D<sub>2</sub> were used to treat tuberculosis in the pre-antibiotic era, with reported success.
- Laboratory studies have elucidated diverse mechanisms by which vitamin D metabolites support innate anti-mycobacterial immune responses.
- Observational epidemiological studies have reported consistent associations between low vitamin D status and increased risk of tuberculosis infection and disease.
- Randomized controlled trials of vitamin D for prevention and treatment of tuberculosis have yielded consistent null results. Consequently, international guidelines do not currently recommend a role for vitamin D supplements in either prevention or treatment of tuberculosis.

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# The role of vitamin D in COVID-19

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## OBJECTIVES

- Present an introduction to COVID-19 and vitamin D.
- Discuss the potential biological mechanisms of vitamin D and COVID-19 infection.
- Review of the results of key observational studies of vitamin D status and COVID-19.
- Present the results of studies and clinical trials of vitamin D supplementation and COVID-19.
- Discussion on current guidance and the areas for future research.

## 1. Introduction

COVID-19 is caused by the novel coronavirus (SARS-CoV-2), thought to originate from a food market in Wuhan, China, in mid-December 2019. COVID-19 has since spread globally, and on March 11, 2020, it was named a pandemic by the WHO, and as of April 3, 2022, there have been an estimated 489 million positive cases and 6 million deaths globally [1]. COVID-19 symptoms include dry cough, fever, breathlessness, sore throat, nasal congestion, loss of smell, and malaise. The severity of COVID-19 infection ranges from asymptomatic, mildly symptomatic, moderate disease requiring hospitalization, and severe and critical requiring need for admission to the intensive care unit (ICU). Multiple risk factors for worse outcomes in COVID-19 include older age, black and minority ethnic groups, obesity, cardiovascular comorbidity, and

malignancy, which are also risk factors for vitamin D deficiency [2]. Therefore, there has been great interest in the role of vitamin D in COVID-19 infection and severity. Since the beginning of the pandemic, there have been an increasing number of genetic variants, which have given an evolutionary advantage of the virus to infect and spread through populations; however, there is a lack of information about the role of vitamin D in the severity of different variants of SARS-CoV-2.

SARS-CoV-2 is an enveloped virus with a positive-sense single-stranded ribonucleic acid (RNA) genome. SARS-CoV-2 contains four structural proteins: spike (S), membrane (M), envelope (E), and nucleocapsid (N). The SARS-CoV-2 virus enters the cell via the spike protein, which has a key role in receptor recognition and cell membrane fusion. The spike protein is comprised of two subunits (S1 and S2). The S1 subunit binds to the enzyme angiotensin-converting enzyme 2 (ACE2) [3], which is present in upper airway epithelial cells in high concentrations [4].

The major causes of morbidity and mortality of SARS-CoV-2 infection are through the development of acute respiratory distress syndrome (ARDS). The initial infection of the respiratory virus results in a phase of viral replication. This is followed by a recruitment of inflammatory cells including neutrophils and monocytes/macrophages from proinflammatory cytokines from viral-infected cells. Resultant cellular apoptosis of lung epithelial cells and endothelial cells causes vascular leakage and alveolar oedema, developing into ARDS.

## 2. Vitamin D and immunity and inflammation: relevance to COVID-19 infection

The vitamin D receptor (VDR) is present in most tissues including immune cells. Vitamin D when bound

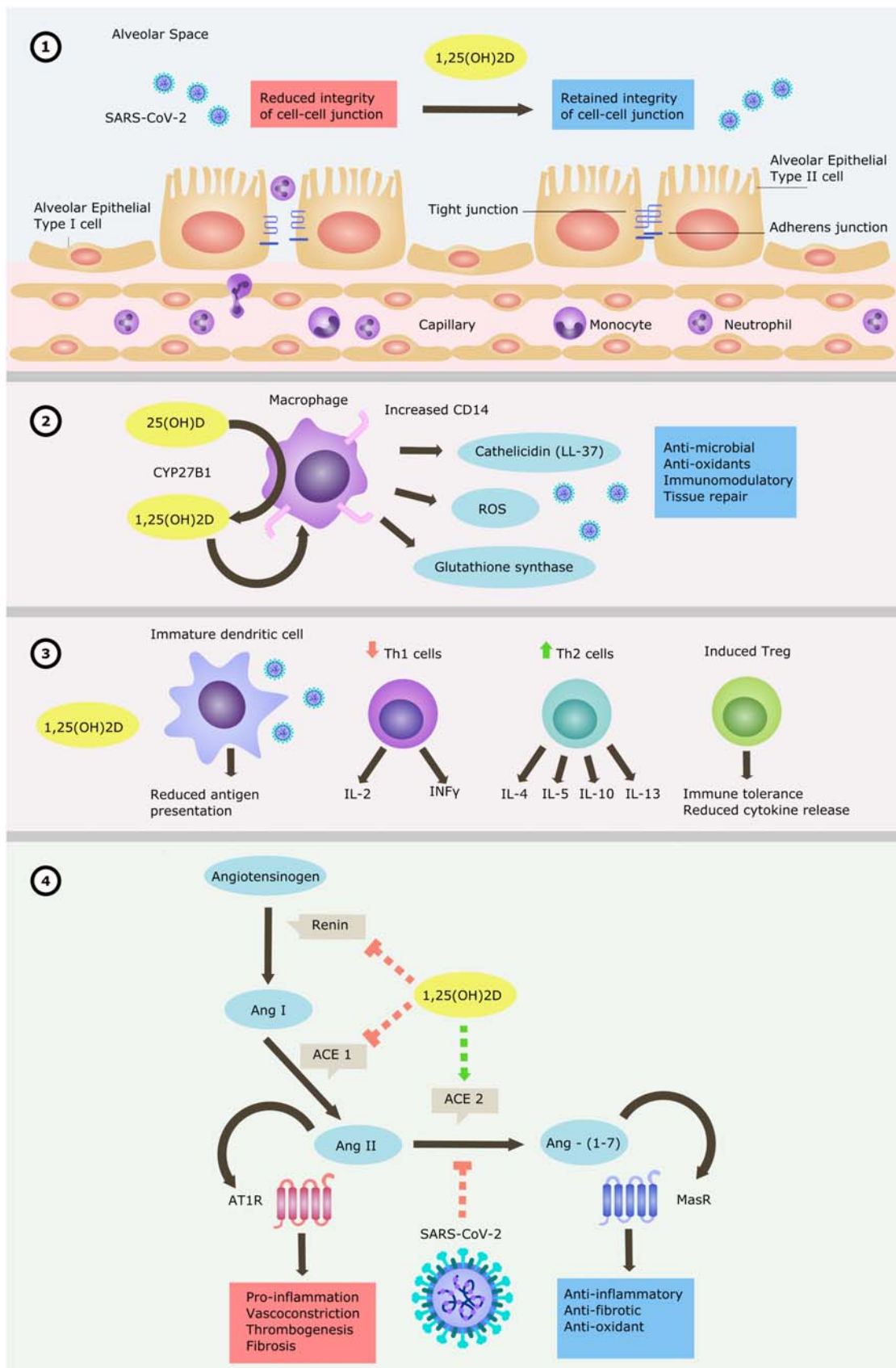
to the VDR can regulate many target genes with effects on immunomodulation of the immune system and lung cell function. Vitamin D has a role in both the innate and adaptive immune system. The proposed mechanisms by which vitamin D can protect/limit the severity of COVID-19 infection are summarized in Fig. 99.1.

The first potential mechanism by which vitamin D can influence the course of COVID-19 infection is via its well-established effects on the immune system. In the innate immune system, the active form of vitamin D, 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ), has a role in antimicrobial activity and limiting inflammation/promoting tissue repair. Antigen-presenting cells (APCs) such as activated macrophages and dendritic cells (DCs) express cytochrome P450 family 27 subfamily B member 1,  $1\alpha$ -hydroxylase (CYP27B1), which converts precursor 25-hydroxyvitamin D ( $25(\text{OH})\text{D}$ ) into  $1,25(\text{OH})_2\text{D}$  [5,6] (see Chapter 9).  $1,25(\text{OH})_2\text{D}$  reduces production and release of proinflammatory cytokines by blocking transcription factor nuclear factor kappa B (NF- $\kappa$ B) translocation to the nucleus [7]. Vitamin D also has a role in monocyte to macrophage differentiation and triggers autophagy needed to defend against viral infection [8].  $1,25(\text{OH})_2\text{D}$  also has a role in microbial killing by activation of Toll-like receptors (TLRs) (see Chapters 94 and 95).  $1,25(\text{OH})_2\text{D}$  induces CD14 [9], which is a TLR coreceptor; subsequent TLR activation leads to induction of reactive oxygen species (ROS) and antimicrobial peptides. The potential of ROS damage is limited by  $1,25(\text{OH})_2\text{D}$  through induction of antioxidants such as glutathione synthase [10].  $1,25(\text{OH})_2\text{D}$  indirectly induces defensin Beta-2 [11], which stimulates antiviral cytokines and chemokines involved in cellular recruitment of innate immune cells.  $1,25(\text{OH})_2\text{D}$  also induces expression of cathelicidin antimicrobial peptide (CAMP or LL-37) in epithelial cells and myeloid cells to promote antibacterial and antiviral immunomodulation and tissue repair [12,13]. In patients with non-COVID-19 sepsis, a single dose of 400,000 IU of vitamin D<sub>3</sub> treatment resulted in an increase in serum cathelicidin [14]. With regard to COVID-19, LL-37 competitively binds to SARS-CoV-2, which inhibits the virus binding to ACE2 and subsequent invasion into the cell [15] (see Chapter 95).

In the adaptive immune system,  $1,25(\text{OH})_2\text{D}$  has an overall inhibitory role by decreasing the maturation of DCs via reduced expression of costimulatory cell surface markers [16] and by decreasing DC antigen expression and subsequent activation of T cells to mount an adaptive response.  $1,25(\text{OH})_2\text{D}$  also inhibits the development of T helper type 1 (Th1) cells, thereby inhibiting the production of interleukin-2 (IL-2) and interferon  $\gamma$  (INF $\gamma$ ), and also suppresses Th17 cells, which produce the inflammatory cytokine IL-17 [17]. In suppressing Th1 cells,  $1,25(\text{OH})_2\text{D}$  promotes the transition of proinflammatory

Th1 cells to suppressive T helper type 2 (Th2) phenotype producing IL-10 [18]; the effects of  $1,25(\text{OH})_2\text{D}$  can occur in the absence of the APC such as DC [19]. Exposure of DCs to  $1,25(\text{OH})_2\text{D}$  results in increased expression of regulatory transcription factor forkhead box P3 (Fox3) [17], and induction of regulatory T cells (Treg), which have an important role in immune tolerance (see Chapter 96). Thus, vitamin D has a role in the shift from a Th1 to Th2 environment, as there is prevention of further recruitment of T lymphocytes and proliferation, and suppression of IL-12 resulting in more Th2, which produces IL-4 (differentiation of Th0 to Th2), IL-5, and IL13. In addition, induction of Treg cells has an important role in preventing/limiting the cytokine/chemokine release associated with ARDS caused by SARS-CoV-2 and other viruses. The role of vitamin in the adaptive immune system has been investigated in patients with COVID-19. This has been shown in a study, which showed that serum  $25(\text{OH})\text{D}$  levels  $\leq 11.4$  ng/mL (vitamin D deficiency) were associated with increased circulating Th2 and decreased Th17 in patients with COVID-19 [20]. Furthermore, in patients with COVID-19, CD4 T cells from bronchoalveolar lavage from the lungs showed a higher proportion of Th1 cells than uninfected controls, and these cells were characterized by derepression of genes that are normally downregulated by vitamin D [18]. Furthermore, Th1 cells from COVID-19 patients were able to convert  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$  and are thus not solely reliant on paracrine effect of  $1,25(\text{OH})_2\text{D}$  produced by macrophages or DCs (see Chapter 9). Thus, the antiinflammatory processes of the adaptive immunity in COVID-19, and other inflammatory diseases, are likely to be compromised in the setting of vitamin D deficiency.

The second potential mechanism associated with COVID-19 mortality that may be impacted by vitamin D is the renin-angiotensin system (RAS). Angiotensin-converting enzyme 2 (ACE2) plays a role in cleaving angiotensin II, thus limiting the RAS. Vitamin D has been shown to increase ACE2 levels, therefore inhibiting RAS [21]. Signal transduction in the RAS is modulated by two pathways, the classical angiotensin II type I receptor (AT1R) axis and the alternative mass receptor (MasR) axis. Vitamin D inhibits the classical pathway while enhancing the alternative pathway [22], promoting antiinflammatory, antioxidative, and antiapoptosis, therefore reducing the effect of angiotensin in inflammation and fibrosis, vasoconstriction, and thrombogenicity. The binding of SARS-CoV-2 to ACE2 subsequently reduces ACE2 levels. Absence of ACE2 in mice results in severe lung injury [23], and RAS has been implicated in the development of ARDS. Polymorphisms of the ACE gene have also been shown to be linked to ARDS, providing further evidence of a role for the RAS in the development of ARDS [24,25].





The final potential target for vitamin D in COVID-19 relates to the lung epithelial permeability is associated with ARDS. Similar to its effects on other barrier tissues such as the gastrointestinal tract (see Chapter 97), Vitamin D plays a key role in maintaining the integrity of tight junctions (TJ) and adherens junctions (AJ) within the pulmonary epithelial barrier. TJ and AJs are significantly impaired in *Vdr* knockout mice [26], which is associated with increased alveolar permeability and more severe lung injury following exposure of lipopolysaccharide, which is present on the cell surface of gram-negative bacteria [27]. Airway epithelial cells express CYP27B1, where  $1,25(\text{OH})_2\text{D}$  has a role in proliferation and reducing apoptosis following inflammation, thus promoting epithelial integrity [26].

### 3. Vitamin D and respiratory tract infections

Vitamin D has previously been shown to reduce the risk of respiratory infections. In a metaanalysis of randomized controlled trials (RCTs) including 10,933 participants, supplementation reduced the risk of one or more acute respiratory tract infections (ARIs) from 42.2% to 40.3% [28]. The benefit was most observed in those who were deficient at baseline (55%–40.5%). An updated version of this metaanalysis included 43 RCTs [29], which concluded that vitamin D was safe and reduced ARI, although the reduction in ARI was modest (odds ratio [OR] of 0.92; 95% CI 0.86–0.99) in 37 studies of 23,364 participants assessed. Furthermore, this benefit was not seen with intermittent dosing, and protection was found in those who were supplemented on 400–1000 international units (IU)/day for 12 months and aged between 1 and 16 years [29].

Further support of a role for vitamin in preventing viral respiratory tract infections has been shown in a study where children supplemented with vitamin D<sub>3</sub> during the winter are less likely to develop influenza A [30]. There is also an association between vitamin D genetics and the severity of respiratory infection. VDR polymorphisms (FokI) resulting in lower transcriptional activity of VDR have been shown to be associated with

severe respiratory syncytial virus (RSV) in infants [31]. Furthermore, in infants with bronchiolitis, severe vitamin D deficiency (serum  $25(\text{OH})\text{D} < 20 \text{ ng/mL}$ ) was associated with higher admission to ICU and longer length of hospital stay [32].

### 4. Vitamin D and COVID-19: geographical latitude and UV radiation

Early in the pandemic, population-based epidemiological studies demonstrated a link between SARS-CoV-2 and geographical latitude. COVID-19 infection cases are fewer and less severe in countries closer to the equator [33], and the mortality of COVID-19 infection increased with northerly latitude after adjustment for age [34]. This is likely due to sun exposure, the natural source of vitamin D (see Chapter 3), suggesting a link between latitude, vitamin D, and COVID-19 risk [35]. For most parts of the globe, this also implies seasonality with regard to vitamin D and COVID-19 [36], as is the case for other respiratory viruses such as influenza and swine flu (H1N1) [37].

Ultraviolet-B (UVB) radiation has also been shown to be associated with reduced COVID-19 mortality [38]. A study from UK Biobank cohort found that ambient UVB measured over the preceding 135 days was inversely associated with hospitalization and death, though it was not associated with COVID-19 infection risk [39]. The mortality rate ratio (MMR) of COVID-19 has been shown to be 32% (95% CI 48%–12%) per  $100 \text{ kJm}^{-2}$  increase in daily mean ultraviolet-A (UVA) across three studies in the United States, England, and Italy [40]. This finding was thought to possibly be mediated via cutaneous nitric oxide release. A further study from the United States, using the Nurse's Health Study II cohort, included 39,315 participants and 1768 testing positive for SARS-CoV-2 infection, with predicted  $25(\text{OH})\text{D}$  levels based on questionnaire data from 2015 [41]. The study found higher predicted circulating  $25(\text{OH})\text{D}$  levels were associated with reduced risk of SARS-CoV-2 infection and hospitalization. In addition, there was an association between UVA and UVB exposure and SARS-CoV-2 infection.

**FIGURE 99.1** Proposed mechanisms of the protective effect of  $1,25(\text{OH})_2\text{D}$  against sars-cov-2 infection. (1)  $1,25(\text{OH})_2\text{D}$  maintains the integrity of tight junctions and adherens junctions within the pulmonary epithelial barrier and reducing alveolar permeability in response to sars-cov-2 infection. (2) conversion of  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$  in the innate immune system promotes antimicrobial activity of alveolar macrophages and limits inflammation/promotes tissue repair. (3)  $1,25(\text{OH})_2\text{D}$  in the adaptive immune system inhibits antigen presentation by dendritic cells and reduces T helper 1 (Th1) cells and promotes Th2 cells and T regulatory cells (treg), thus reducing proinflammatory cytokine release associated with COVID-19 infection. (4)  $1,25(\text{OH})_2\text{D}$  is involved in the renin–angiotensin system (RAS) by cleaving angiotensin II (ang II) and increasing angiotensin II enzyme, promoting systemic antiinflammatory, antifibrotic, and antioxidant pathways, and reducing vasoconstriction and thrombogenesis.

## 5. Vitamin D and COVID-19: community observational studies

There have been many observational studies that have assessed association between vitamin D and COVID-19 infection, severity, and mortality. The characteristics of selected studies investigating vitamin D deficiency and COVID-19 are shown in Table 99.1. Earlier observational studies during the pandemic used historic vitamin D results to investigate this link. A study investigating the mean 25(OH)D levels and the cases/deaths of COVID in 2020 found there was a negative correlation between mean 25(OH)D levels and infection rate and mortality [42]. Another population-based study in the United States in 2020 including 191,779 participants demonstrated that SARS-CoV-2 positivity rates were inversely correlated to circulating 25(OH)D levels from the preceding 12 months [43]. A UK Biobank study of 348,958 participants, including 449 patients with COVID-19 infection and historic 25(OH)D levels between 2006–10, found no increase in 25(OH)D levels in multivariate analysis [44]. A follow-up study of this data including in 341,484 participants found, again, no association with COVID-19 infection and mortality after adjustment for multiple variables including body mass index (BMI) and ethnicity [45]. However, the biobank studies are severely limited in drawing conclusions due to the use of serum 25(OH)D levels obtained up to 10 years prior to COVID analysis.

A single-center retrospective cohort study from the United States in 2020 looked at COVID-19 infection and vitamin D status within the previous year ( $<20$  ng/mL) including 489 participants [46]. Those with likely vitamin D deficiency ( $<20$  ng/mL) had an associated increased COVID-19 risk on multivariate analysis compared with those who were likely sufficient (1.77; 95% CI 1.12–2.81;  $P = .02$ ). A retrospective study in Israel in 2020, which included 7807 people who had tested for COVID-19 and had previous 25(OH)D levels performed (not specified when), found that low plasma 25(OH)D ( $<30$  ng/mL) was an independent risk factor for COVID-19 infection (OR 1.45; 95% CI 1.08–1.95,  $P < .001$ ) and hospitalization (OR 1.95; 95% CI 0.98–4.845,  $P = .061$ ) [47]. Another retrospective study of 2020 veterans in the United States, 2020 veterans included patients with positive SARS-CoV-2 and blood test within 15–90 days also found inverse relationship with mortality and severity of COVID-19 infection [48].

There have been a number of studies, which have looked at the association of ethnicity, vitamin D deficiency, and COVID-19. One of which used propensity score methods to examine the effect of ethnicity on the relationship between vitamin D status and COVID-19 test positivity using electronic health data

from unmatched ( $n = 21,629$ ) and matched ( $n = 16,602$ ) with testing of 25(OH)D levels [49]. The study found deficiency ( $<30$  ng/mL) was not associated with COVID-19 positivity overall, though when analyzed as a continuum, 25(OH)D levels  $<10$  ng/mL lowered odds of testing for COVID-19 in those of white (OR 0.935;  $P = .003$ ) and not black ethnicity (OR 0.994;  $P = .75$ ). In contrast to these findings, another study investigated COVID-19 infection in 13,000 participants of the US Black Women's Health Study who provided blood samples between 2013– and 2017; these were subsequently analyzed for 25(OH)D in addition to online questionnaire [50]. In 5081 eligible participants, the study found that black women with lower 25(OH)D levels were at increased risk of COVID-19 infection on multivariate analysis (20–29 ng/mL; OR 1.48; 95% CI 0.95–2.30;  $<20$  ng/mL; OR 1.69; 95% CI 1.04–2.72). Another study looking at UK healthcare workers who self-isolated with symptoms during the first wave of the pandemic [51] half of who had seroconverted found that seroconversion was increased in those who were vitamin D deficient compared with non-vitamin D-deficient staff (72% vs. 51%). Black, Asian, or minority ethnicity (BAME) or vitamin D deficiency ( $<30$  nmol/L) were independent factors of being positive for SARS-CoV-2 antibodies (indicating previous COVID-19 infection). Thus, ethnicity appears to have a role in risk of developing COVID-19, where vitamin D deficiency is more common, though vitamin D deficiency was an independent risk factor for seropositivity (OR 2.6; CI 1.41–4.8;  $P = .002$ ).

For patients who are hospitalized for COVID-19, there are several treatments that can be used, which have been shown to improve outcomes. The main treatment involves either antiviral therapy or antiinflammatory treatment. Remdesivir was the first antiviral to be approved for treatment, with mechanism of an RNA-dependent RNA polymerase inhibitor, which has an inhibitory effect of SARS-CoV-2 and has been shown to shorten recovery time in patients hospitalized with COVID-19 [59]. Dexamethasone has an antiinflammatory role, and in those hospitalized for COVID-19, has been shown to reduced mortality in those requiring oxygen therapy [60] and now is widely used in clinical practice. There have since been monoclonal antibody therapies used in patients with COVID-19, which have antiinflammatory effects. Anti-IL6 therapies, such as tocilizumab, have been shown to improve survival in patients with hypoxia and systemic inflammation [61]. Further therapies such as baricitinib has a role in patients hospitalized with COVID-19 requiring supplemental oxygen and suppresses the JAK-STAT pathway, preventing proinflammatory cytokine release and systemic inflammation, and has been shown to reduce 28-day mortality in hospitalized patients [62]. Sotrovimab,

**TABLE 99.1** Characteristics of selected studies investigating vitamin D deficiency and COVID-19.

Study, year	Country	Design	Sample size	Definition of VitD deficiency	Findings
Ma et al. [41]	United States	Retrospective cohort study	39,315	N/A	Higher predictive 25(OH)D levels were associated with lower risk of SARS-cov-2 infection.
Ilie et al. [42]	20 european countries	Retrospective population study	N/A	N/A	Mean levels of vitamin and morbidity and mortality for COVID-19 acquired for each country. Negative correlation found between mean levels of vitamin D and COVID-19 infection and mortality.
Kaufman et al. [43]	United States	Retrospective cohort study	191,779	<20 ng/mL	Data from 50 states showed SARS-cov-2 positivity inversely related 25(OH)D levels.
Hastie et al. [44]	United Kingdom	Retrospective cohort study	348,958	N/A	Using historical vitamin D levels (2006–10). There was a significant association of VitD and COVID-19 infection on univariate analysis; not on multivariate analysis after adjustment of confounders.
Hastie et al. [45]	United Kingdom	Retrospective cohort study	341,484	N/A	Using historical vitamin D levels (2006–10). There was no association with COVID-19 infection and mortality after adjustment for multiple variables including BMI and ethnicity.
Meltzer et al. [46]	United States	Retrospective cohort study	489	<20 ng/mL	Likely VitD deficiency associated with increased COVID-19 infection on multivariate analysis.
Merzon et al. [47]	Israel	Retrospective population study	7807	<30 ng/mL	Previously low plasma 25(OH) D, an independent risk factor of COVID-19 infection and hospitalization.
Seal et al. [48]	United States	Retrospective cohort study	4599	<20 ng/mL	Inverse relationship with mortality and severity of COVID-19 infection found.

Crandell et al. [49]	United States	Retrospective propensity-matched study	21,629 unmatched 16,602 matched	<30 ng/mL	VitD deficiency was not associated with COVID-19 positivity: Though when analyzed as a continuum, vitamin D levels 10 ng/mL lowered odds of testing for COVID-19 in those of white and not black ethnicity.
Cozier et al. [50]	United States	Retrospective cohort study	5,081 participants	<30 ng/mL	Using historic blood samples (2013–17) and survey data, black women with lower 25(OH)D levels were at increased risk of COVID-19 infection on multivariate analysis.
Faniyi et al. [51]	United Kingdom	Cross-sectional observational study	392	<30 nmol/L	VitD deficiency was independently associated with SARS-cov-2 seropositivity.
Israel et al. [52]	Israel	Retrospective case-control study	41,757 COVID-19 cases, 417,570 non-COVID-19 controls 2533 COVID-19 hospitalized cases, 2533 COVID-19 not-hospitalized controls	<30 ng/mL	VitD deficiency was associated with higher risk for infection and severe disease.
Hernandez et al. [53]	Spain	Retrospective case-control study	216 COVID-19 hospitalized cases; 197 population controls	<20 ng/mL	25(OH)D levels lower in COVID-19 cases and inversely correlated with inflammatory markers serum ferritin and D-dimer. There was no association between VitD deficiency and severity of disease.
Radujkovic et al. [54]	Germany	Prospective cohort study	185	<12 ng/mL	VitD deficiency associated with severity of infection (requiring invasive mechanical ventilation) and mortality.
Carpagnano et al. [55]	Italy	Retrospective cohort study	42	<10 ng/mL	Severe VitD deficiency in COVID-19 patients admitted to ITU was associated with higher mortality risk.
Jain et al. [56]	India	Prospective cohort study	154	<20 ng/mL	25(OH)D levels were lower in severe COVID-19 infection. VitD deficiency associated with higher levels of inflammatory markers and increased mortality.

Continued



**TABLE 99.1** Characteristics of selected studies investigating vitamin D deficiency and COVID-19.—cont'd

Study, year	Country	Design	Sample size	Definition of VitD deficiency	Findings
Hurst et al. [57]	United Kingdom	Cross-sectional study	295 hospitalized COVID-19 patients, 93 patients with influenza A, 139 non-COVID-19 critical illness survivors	<25 ng/mL	VitD deficiency seen in majority of hospitalized cases of COVID-19 or influenza A, correlated with severity, and persisted in those of non-covid-19 critical illness survivors.
Subramanian et al. [58]	United Kingdom	Retrospective cohort study	472	<50 ng/mL	In hospitalized patients with COVID-19, a U-shaped associated between 25(OH)D levels and mortality; <25 nmol/L and >100 nmol/L was associated with increased risk.

25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; ICU, intensive care unit.

a neutralising monoclonal antibody, directed against the spike protein, with a role for treatment to nonhospitalized high-risk patients with mild to moderate COVID-19 [63]. Vitamin D also shares similar mechanisms with regard to antiviral (prevention) and antiinflammatory effects (treatment) of COVID-19 and therefore could be considered as a concomitant treatment in prevention and/or treatment.

## 6. Vitamin D and COVID-19: hospital observational studies

For those who are hospitalized due to COVID-19 infection, studies have investigated the role of vitamin D in relation to infection and severity of disease. An Israeli retrospective study in 2020 included 41,757 individuals who tested positive for COVID-19 matched against 417,570 non-COVID-19 controls; another 2533 who were hospitalized with COVID-19 were matched against 2533 patients who tested positive but were not hospitalized [52]. The study found an inverse correlation between 25(OH)D levels and developing COVID-19 infection and being hospitalized with infection. Those with lowest levels ( $<30$  nmol/L) had highest risk of infection (OR 1.246; 95% CI 1.21–1.30) and severe disease (OR 1.513; 95% CI 1.22–1.86). A retrospective case–controlled Spanish study included 216 hospitalized patients with COVID-19, and 197 population controls found that 25(OH)D levels were lower in COVID-19 cases (82.2% versus 47.2%) [53]. Furthermore, 25(OH)D inversely correlated with acute phase inflammatory markers (ferritin) and D-dimer (associated with coagulopathy). This study, however, did not find significant differences between vitamin D deficiency and severity of COVID-19 infection. In contrast, a prospective study in Germany assessed 25(OH)D levels at presentation for COVID-19 in 185 patients. There was an association between vitamin D deficiency (defined as  $<12$  ng/mL ( $<30$  nmol/L) serum 25(OH)D) and COVID-19 severity (requiring invasive mechanical ventilation) and mortality [54].

Those patients with severe COVID-19 with ARDS require admission to ICU for ventilatory support; a retrospective observational study in patients with COVID-19 in ICU found 81% of patients were vitamin D deficient ( $<10$  ng/mL ( $<25$  nmol/L) serum 25(OH)D), and these patients had 50% mortality risk compared with those with 25(OH)D levels  $\geq 10$  ng/mL (5% mortality risk) [55]. This suggested that vitamin D deficiency is associated with poorer prognosis in these individuals. A further study of COVID-19 patients aged 30–60 years ( $n = 154$ ) found that vitamin D

deficiency was higher in those with severe disease (requiring ICU) compared with asymptomatic cases (96.82 vs. 32.69%) [56]. Vitamin D–deficient patients had increased levels of acute phase inflammatory markers (IL-6, ferritin, and tumor necrosis factor  $\alpha$  [TNF $\alpha$ ]) and increased mortality.

A UK cross-sectional study measured plasma for 25(OH)D concentrations from 295 patients hospitalized with COVID-19, 93 patients with influenza A, and 139 survivors from nonselected critical illness prior to the pandemic [57]. The study found that vitamin D deficiency was observed in the majority of hospitalized cases of COVID-19 or influenza A, and that vitamin D deficiency correlated with disease severity, and persisted in those of non-COVID-19 critical illness survivors. There therefore may not be a causal role for vitamin D deficiency in COVID-19 severity, as serum levels of 25(OH)D have been reported to be a negative acute phase reactant [64,65]. Therefore, lower serum levels of 25(OH)D might occur as a consequence of more severe systemic disease rather than be a cause of this disease. A reduction in 25(OH)D levels has been seen in other acute illnesses such as acute pancreatitis and following surgery [65]; a possible biological explanation may be due to the fact the majority of 25(OH)D is bound to vitamin D–binding protein (DBP) and serum albumin, which both decrease during acute illness [66].

To investigate further the link between 25(OH)D status and COVID-19 disease severity, a UK study assessed mortality of 472 patients hospitalized for COVID-19 [58]. The authors found a U-shaped association between total 25(OH)D levels and mortality, with levels  $<25$  nmol/L and  $>100$  nmol/L associated with increased mortality (OR 2.37; 95% CI 1.17–4.78 and OR 4.65; 95% CI 1.51–14.34, respectively). The higher risk seen in those with high levels should be approached with caution as the numbers with levels  $>100$  nmol/L were very low ( $n = 18$ ). There were, however, no differences found between either free or bioavailable 25(OH)D and risk of death. Therefore, the relationship between COVID-19 and vitamin D is difficult to be explained by the onset of acute illness alone. There are two recent metaanalyses on this question with divergent conclusions. In identifying 31 observational studies, one systematic review found a trend for an association between low serum 25(OH)D levels and COVID-19-related outcomes, but the relationship was not statistically significant [67]. Another review looked at 17 observation studies finding that vitamin D deficiency was associated with greater severity of COVID-19 [68]. Thus, further research is needed to determine if vitamin D supplementation can reduce the severity of COVID-19.

## 7. Vitamin D and COVID-19: effects of polymorphisms in VDR and vitamin D-binding protein genes

As detailed in other chapters in this book (see Chapters 60 and 61), single-nucleotide polymorphic (SNP) variations in genes encoding key components of the vitamin D system play a crucial role in modulating the vitamin D metabolism, transport, and function. However, while these vitamin D SNPs have been linked to many other human diseases, there have been a few studies that have investigated the impact of vitamin D SNPs on COVID-19. One study found that serum 25(OH)D levels were not related to COVID-19 outcomes, but SNPs in the *VDR* gene were independently associated with COVID-19 severity and survival [69]. A Portuguese study showed that a polymorphism in the *DBP* gene (*GC*), referred to as RS2282679, was associated with severity of COVID-19 infection [70]. *DBP* not only has a role in transporting vitamin D metabolites in the circulation but also has an indirect role as a neutrophil chemotactic factor [71], alveolar macrophage activation, and systemic actin binding, indicating a broader role in the inflammatory process [72] (see Chapter 7). *DBP* is particularly important in relation to 25(OH)D levels in severely ill COVID-19 patients with ARDS; circulating levels of *DBP* have been shown to drop by about a third in ARDS [73]. However, because *DBP* is the main serum binder of 25(OH)D, a drop in *DBP* levels may, indirectly, lead to an increase in free 25(OH)D concentration, which may enhance the availability of 25(OH)D for immune cells, notably APC (see Chapter 7). The relationship between *GC* variants, *DBP* levels, and 25(OH)D function is complex, and SNPs contribute a relatively small proportion of overall vitamin D variability. Thus, the role of *DBP*/vitamin D homeostasis in the setting of COVID-19 is still unclear.

## 8. Vitamin D and SARS-CoV-2 vaccination response

As well as assessing the impact of 25(OH)D status on COVID-19 infection and disease severity, studies have also investigated the effect of vitamin D on immune response following SARS-CoV-2 vaccination. A study looked at 97 healthcare workers following a first dose of vaccine (BNT162b2) included serum 25(OH)D measurements at baseline [74]. The study found that in individuals with 25(OH)D > 50 nmol/L, there was on average a 29.3% greater peak value of antibody response. In contrast, a German study looked at the SARS-CoV-2 IgG antibody and neutralization potency in association with 25(OH)D concentrations in healthy

adults [75]. The study found no association between vitamin D status and vaccination response as measured as SARS-CoV-2 IgG. In addition, vitamin D replacement at a dose of 800 or 3200 IU/day did not influence the protective efficacy or immunogenicity of the SARS-CoV-2 vaccination when given to adults who had suboptimal vitamin D status at baseline [76]. Thus, currently, there is conflicting evidence of the relationship between vitamin D status and SARS-CoV-2 vaccination response. A previous metaanalysis of influenza vaccination and serum 25(OH)D found no difference between vitamin D levels and immunogenic response following vaccination [77]. However, a strain-specific differences may exist, for example, the seroprotection rates of influenza A subtype and B strain in vitamin D-deficient patients were lower than in patients with normal vitamin D levels.

## 9. Vitamin D supplementation and COVID-19

Following observational data on the association of vitamin D deficiency to COVID-19 infection, severity, and mortality, the effects of supplementation have been investigated and summarized in Table 99.2. A Spanish observational study included 15,968 hospitalized COVID-19 patients from the region of Andalusia between January to November 2020 [78], including those who were prescribed vitamin D prior to admission. The study found a relationship between patient survival and vitamin D replacement within 15 days; higher in those who had calcifediol (HR 0.67; 95% CI 0.50–0.91) than cholecalciferol (HR 0.75; 95% CI 0.61–0.91), and stronger association in those who had this within 15 days compared with 30 days prior to hospitalization. A French quasi-experimental study included 66 nursing home residents. The intervention ( $n = 57$ ) of a bolus of vitamin D supplementation of 80,000 IU vitamin D was associated with less severe disease and better survival than the comparator group ( $n = 9$ ) [79].

Several RCTs have assessed the effect of vitamin D supplementation. A Mexican study included 42 outpatients who had asymptomatic or mildly symptomatic COVID-19 infection [80]; 22 were randomized to receive 10,000 IU vitamin D for 14 days, the remaining 20 patients were control. After 7 and 14 days of follow-up, the supplemented patients presented more symptoms of fever compared with those who were not supplemented. The SHADE study from India randomized patients to receive 60,000 IU vitamin D for 7 days in asymptomatic or mildly symptomatic individuals with 25(OH)D levels <20 ng/mL with  $n = 16$  in the intervention and  $n = 24$  in the control [81]. The study found that fibrinogen levels were significantly reduced in the vitamin D-treated group; furthermore, an increased

**TABLE 99.2** Characteristics of selected studies investigating vitamin D supplementation/treatment and COVID-19.

Study, year	Country	Design	Sample and group size	Treatment	Findings
Loucera C et al. [78]	Spain	Retrospective cohort study	15,968	Prescription of vitamin D replacement within 15–30 days of admission	In COVID-19 hospitalized patients, vitamin D replacement was associated with survival, higher in those received therapy in 15 days and in those who had calcifediol than cholecalciferol.
Nogues et al. [92]	Spain	Prospective cohort study	838 total 447 treated 391 not treated	Prescription of calcifediol 532 µg on day 1 plus 266ug on day 3, 7, 15, and 30	Reduced ICU admission in the treated group, including on adjusted logistic regression analysis. Increased mortality in the nontreated group.
Annweiler et al. [79]	France	Quasi-experimental	66 total 57 treated 9 control	800,000 IU oral bolus in the week of diagnosis or in previous month verses untreated control	In nursing home residents, vitamin D treatment was associated with less severe disease and better survival.
Sanchez-Zuno et al. [80]	Mexico	RCT	42 total 22 treated 20 control	10,000 IU/day for 14 days verses untreated control	In outpatients with asymptomatic or mildly symptomatic COVID-19 infection, immunomodulatory effects of vitamin D were linked to COVID-19 symptoms.
Rastogi et al. [81]	India	RCT	40 total 16 treated 24 control	60,000 IU/day of cholecalciferol for 7 days versus placebo control	In asymptomatic/mildly symptomatic COVID-19-positive vitD deficient (<20 ng/mL), treatment reduced fibrinogen levels, and increased proportion became PCR negative.
Lakkireddy et al. [82]	India	RCT	88 total 44 treated 43 control	60,000 IU/day for 8–10 days (depending on BMI) versus untreated control	Vitamin D treatment was associated with reduction in inflammatory makers.
Elamir et al. [83]	United States	RCT	50 total 25 treated 25 control	0.5 µg calcitriol for 14 days or hospital discharge versus untreated control	No difference in LOS, ITU admission, or mortality. Improvement of oxygenation status at discharge in the treatment group.
Murai et al. [84]	Brazil	RCT	240 total 120 treated 120 control	200,000 IU single-dose oral vitamin D (n = 60) or placebo (n = 60)	In hospitalized patients with moderate/severe COVID-19, no difference in clinical outcomes including LOS.

*Continued*



**TABLE 99.2** Characteristics of selected studies investigating vitamin D supplementation/treatment and COVID-19.—cont'd

Study, year	Country	Design	Sample and group size	Treatment	Findings
Cannata-Andia et al. [85]	Spain	RCT	543 total 274 treated 269 control	Oral bolus of cholecalciferol 100,000 IU versus untreated control	In moderate–severe COVID-19, hospital LOS, ITU admission and mortality did not differ between groups. In the cohort analysis, higher serum 25(OH)D at admission (>25 ng/mL) was associated with better outcomes.
Maghbooli et al. [86]	Iran	RCT	106 total 53 treatment 53 control	25 µg calcifediol once/day (3000–6000 IU/day equivalent) for 60 days versus placebo control	In hospitalized patients with vitD deficiency (<30 ng/mL), treatment was associated with increase in lymphocyte percentage and NLR but no difference in LOS and hospitalization, ITU, or mortality.
Entrenas castillo et al. [87]	Spain	Pilot RCT	76 total 50 treatment 26 control	Oral calcifediol 0.532 mg on admission; 0.266 mg on day 3 and 7 and then weekly until ITU admission versus untreated control	Increase admission to ITU in untreated group and no deaths in treated group.
Sabico et al. [88]	Saudi Arabia	RCT	69 total 36 treatment A 33 treatment B	5000 IU/day (A) versus 1000 IU/day (B) for 2 weeks	Increased dose treatment associated shorter time for recovery and return of sense of smell
Joliffe et al. [89]	United Kingdom	RCT	6200 total 1550 treatment A 1550 treatment B, 3100 control	Using test and treat approach for 25(OH)D levels <75 nmol/L; 6 months of either 3200 IU/day (A) or 800 IU/day (B), or no offer or test/treatment	Test and treat approach did not reduce risk of all-cause ARI.

*IU*, international unit; *RCT*, randomized controlled trial; *25(OH)D*, 25-hydroxyvitamin D; *BMI*, body mass index; *PCR*, polymerase chain reaction; *NLR*, neutrophil lymphocyte ratio; *LOS*, length of stay; *ICU*, intensive care unit; *ARI*, acute respiratory infection.

proportion in the treated group became SARS-CoV-2 negative on polymerase chain reaction (PCR) testing.

Another RCT from India investigated supplementation in patients with vitamin D deficiency (25(OH)D < 30 ng/mL) with mild to moderate COVID-19 infection. The patients were given 60,000 IU/day vitamin D for 8–10 days depending on BMI, versus standard treatment controls ( $n = 44$  and  $n = 43$ , respectively, completed the study) [82]. The study found an improvement in inflammatory markers, blood neutrophil:lymphocyte ratio (NLR), C-reactive protein (CRP), lactate dehydrogenase (LDH), IL-6, and ferritin in patients who were treated compared with baseline, and between treated and untreated groups. An RCT from Saudi Arabia included patients with mild to moderate COVID-19 to receive once-daily 5000 IU ( $n = 36$ ) or 1000 IU of vitamin D ( $n = 33$ ) for 2 weeks in patients with suboptimal vitamin D status. Increase in vitamin D dose to 5000 IU/day was associated with shorter time for recovery of cough and sense of smell. An RCT from the United States included 0.5  $\mu$ g of calcitriol (1,25(OH)<sub>2</sub>D) daily for 14 days or hospital discharge. Including 25 patients in both arms, this study found improvement in oxygenation at discharge in the calcitriol-treated patients, but this was a pilot study, and patient numbers were small [83].

An RCT from Brazil included 240 patients hospitalized with moderate to severe COVID-19 who received 200,000 IU single oral dose of vitamin D<sub>3</sub> dissolved in a 10 mL peanut oil solution ( $n = 60$ ) or placebo ( $n = 60$ ); no differences in outcomes observed including no difference in length of stay (LOS) [84] and did not include only deficient/insufficient patients. Also, in this study, patients only received vitamin D treatment after several days in hospital, and the effects were studied for a relatively short time (7–10 days), which may not be enough time to raise the serum 25(OH)D to sufficient levels. An international multicenter RCT named COVI-VIT-D investigated a single oral bolus of 100,000 IU at hospital admission in patients with moderate to severe COVID-19 disease and found no differences in outcomes between groups. It is important to recognize that while large bolus doses of vitamin D can successfully elevate serum levels of 25(OH)D, this may be against the backdrop of superinduction of the vitamin D catabolic system (induction of the enzyme CYP24A1, for example) that may rapidly inactivate any 1,25(OH)<sub>2</sub>D that is generated [28]. In the cohort analysis, higher serum calcifediol levels at admission were associated with better outcomes [85]. An Iranian study included patients with deficiency/insufficiency (<30 ng/mL 25(OH)D) treated with a dose of 25  $\mu$ g (1000 IU)/day oral vitamin D. This treatment increased lymphocyte percentage and neutrophil lymphocyte ratio (NLR) but had no significant effect on LOS and hospitalization,

ICU, or mortality [86], though higher prevalence of patients with diabetes mellitus hypertension and obesity in the vitamin D-treated group. Lower NLR was associated with reduced ICU LOS and mortality. Higher NLR and lower lymphocyte to CRP ratio has been shown in patients with COVID-19 [90].

An open-label RCT from Cordoba in Spain including 76 hospitalized COVID-19 patients investigated the effect of 25-hydroxyvitamin D (calcifediol) treatment 0.532 mg on the day of hospital admission, 0.266 mg on day 3 and 7, and then weekly until ICU admission [87]. Calcifediol does not need to undergo 25 hydroxylation in the liver unlike regular vitamin D (cholecalciferol) and is thus elevated more rapidly in serum [91]. Of the 50 patients on treatment, only 1 was admitted to ICU, while 13 of 26 in the untreated group required admission. There were no deaths in the treated group, while there were two in the untreated group. However, the authors did not state the 25(OH)D levels at baseline. The lack of blinding in the treatment group, the difference in comorbidities between comparison groups and use of antiinflammatory in standard care which would not be considered standard practice limits generalisability of these finding. There are also larger follow-up studies in Spain in which calcifediol was used, including observational cohort studies that found those treated with calcifediol had reduced ICU admission following adjustment on logistic regression [92], and a reduction in in-hospital mortality [93].

A UK-based RCT CORONAVIT NCT04579640 has recently been published [89], which randomized 6200 adults to receive finger prick analysis of serum 25(OH)D, with provision of 6 months of treatment to those with 25(OH)D levels <75 nmol/L (test and treat approach) versus a control group ( $n = 3100$ ) with no offer of testing/treatment but who were allowed to follow the UK governmental guidance of supplementation with 400 IU/day vitamin D. The treatment was open-label and was either higher-dose daily vitamin D replacement (3200 IU/day;  $n = 1550$ ) or lower dosing (800 IU/day;  $n = 1550$ ). The study found no differences in acute respiratory infections and development of COVID-19 between the groups. However, the study to detect COVID-19 was underpowered, as due to effect of public health measures and vaccinations (89.1% had received at least one dose of COVID-19 vaccination by the end of the study), the proportion of those who became infected were only 4.6%, whereas it was initially powered based on predicted 20% in the sample size calculations. In addition, the control group at the end of the study showed a healthy increase in mean 25(OH)D levels of 66.6 nmol/L, and approximately half of the group took vitamin D supplementation during the study period, thus making it more difficult to draw comparisons on the effect of vitamin D replacement in this study.

There have been a number of metaanalyses assessing the role of vitamin D in COVID-19 infection. One meta-analysis [94] included studies up to November 2020 and included 39 studies of which there were 2 RCTs [81,87] and 1 quasi-experimental study [79]. The metaanalysis found that in the vitamin D-deficient patients, there was higher risk of SARS-CoV-2 infection and a higher degree of severity of infection in the 15 studies addressed: even when adjusting for confounders. Another metaanalysis included 17 observational studies and found that in 2756 patients assessed in total, vitamin D deficiency was associated with a higher mortality, ICU admission, and hospital LOS [68]. A systematic review using 13 observational studies found a positive relationship between vitamin D deficiency and risk of COVID-19 in-hospital mortality (OR 2.11; 95% CI 1.03–4.32); pooled data from five studies showed an inverse association between serum vitamin D and risk of in-hospital mortality (OR 0.94; 95% CI 0.89–0.99). Further Cochrane review [95], identified three RCTs [84,85,87], though overall outcome is limited by differences both clinically and methodologically. Further systematic reviews have been published since [96–99]. A further systematic review has found vitamin D supplementation was associated with reduced risk of ICU admission (RR = 0.35; 95% CI 0.20–0.62) and mortality (RR = 0.46; 95% CI 0.3–0.7), whereas no significant impact on the risk of COVID-19 infection [100]. A further metaanalysis found that in observational studies involving nearly 2 million adults, vitamin D deficiency/insufficiency increased susceptibility to COVID-19 and severe disease, although noting a high risk of bias and heterogeneity in studies, and the association of mortality was less robust [101].

## 10. Current guidance of COVID-19 and vitamin D and future research

The serum levels of 25(OH)D required for optimal human health is a topic that continues to be debated and is the focus of two chapters in this book (Chapters 51 and 52). Widely varying target levels for serum 25(OH)D have been recommended by the Endocrine Society [102], Institute of Medicine [103], and the Science Advisory Council for Nutrition (SACN) in the United Kingdom [104]. These levels of 25(OH)D are based on the classical actions of vitamin D and skeletal health, and it is important to recognize that the optimal levels of 25(OH)D and vitamin D supplementation required to achieve this have yet to be determined for extraskeletal actions of vitamin D. Consequently, there is currently no specific guidance on the serum to prevent or treat COVID-19. In the United Kingdom, guidelines encourage people to take vitamin D supplementation to maintain muscle and bone health at a dose of 400 IU

a day for adults [105], and vitamin D is not offered as supplement solely to prevent or treat COVID-19 unless part of a clinical trial. The Endocrine Society's Practice Guidelines for vitamin D replacement is to aim for sufficiency (serum 25(OH)D > 30 ng/mL) [102]. The majority of observational studies had used this as a cutoff to define vitamin D deficiency and found association with levels of 25(OH)D < 30 ng/mL (75 nmol/L) and increased risk of infection and adverse outcomes relative to those with 25(OH)D levels >30 ng/mL. Therefore, preventing/treating vitamin D deficiency based on this target level may have additional benefits against COVID-19.

There is a potential use of vitamin D in two settings. The first is as a nutritional supplement in the community setting to possibly prevent COVID-19 infection and subsequent severity of disease. The other use of cholecalciferol or calcifediol as treatment for patients who already have COVID-19 infection to improve outcome in this setting. Both represent the two sides to vitamin D function with antiviral mechanisms and antiinflammatory mechanisms. Current RCTs alone have not shown definitive benefit of vitamin D treatment in COVID-19 and preventing acute respiratory infection. The studies vary greatly in methodology and are potentially confounded by many of the other public health measures, vaccination response, and treatments during the pandemic. There are further ongoing studies that have yet to report. These include the COVIT-TRIAL with a primary end point of mortality, aiming to recruit 260 patients at/over 65 years of age; those with high-risk COVID-19 (age over 75 or low oxygenation) having no prior vitamin treatment in last month would be randomized to receive  $2 \times 200,000$  IU or 50,000 IU oral drinks [106]. There is also the VIVID Trial, a pragmatic household cluster randomized trial of vitamin D treatment in COVID-19 for 4 weeks of 3200 IU/day, which has a primary outcome of hospitalization and mortality [107]. There is also the VitCov Trial in a Swiss randomized double-blinding placebo controlled multicenter using high-dose vitamin D 140,000 IU [108]. Finally, the CARED-TRIAL in Argentina is planning to treat hospitalized COVID-19 patients with 50,000 IU with at least one risk factor with primary outcome of respiratory Sepsis-related Organ Failure Assessment score (SOFAr) [109].

## 11. Conclusions

In summary, vitamin D deficiency may contribute to SARS-CoV-2 infection through dysregulated innate and adaptive immune response and through upregulated renin-angiotensin system and inflammatory downstream effects. There are multiple ecological

studies showing a link between latitude, UV exposure, 25(OH)D levels, and COVID-19. This has prompted many community and hospital observational studies, which have shown an association between vitamin D deficiency and infection, morbidity, and mortality, and which are supported in the pooled data from metaanalysis. There have been several studies, which have investigated the effect of treatment in the context of COVID-19, though these differ in the population assessed (community or hospital), severity of disease (asymptomatic/mild or moderate/severe), and treatment regime used (bolus or daily regime). None have shown a difference in clinical outcomes other than one pilot RCT, which had methodological flaws. There is therefore insufficient evidence to recommend vitamin D supplement solely for prevention or treatment of COVID-19. There are further RCTs that seek to investigate further, and more understanding on mechanisms of vitamin-D in COVID-19 infection may help direct further therapy to prevent/ameliorate disease.

## 12. Summary points

- There are two different uses for vitamin D in acute respiratory infection, as a nutrition supplement in prevention and as a potential drug for treatment.
- There are a number of mechanisms by which vitamin D could have both a protective antiviral effect and an antiinflammatory effect in limiting the severity of COVID-19 infection.
- Observational data have shown an association between vitamin D status and COVID-19 infection rates, severity, and mortality.
- Several studies have investigated the effect of vitamin D nutritional supplementation on COVID-19; those showing a benefit on clinical outcomes have significant methodological flaws with larger RCTs showing no clear benefit.
- There is currently insufficient evidence to recommend overall vitamin D supplementation solely for prevention of COVID-19; however, the data on use of calcifediol in particular for treatment in improving the outcome of COVID-19 is encouraging.
- Further RCTs seek to investigate effect of vitamin D supplementation on COVID-19.

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## Further reading

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# Vitamin D and type 1 diabetes

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## OBJECTIVES

- Present the association between vitamin D deficiency and type 1 diabetes.
- Describe the relation between vitamin D–related genes and the genetic predisposition to type 1 diabetes.
- Discuss the influence of vitamin D on the beta cell and the immune system in both animal models and human trials and describe the underlying mechanism of action.
- Describe the current vitamin D analogs used in type 1 diabetes.
- Discuss the role of vitamin D in combination therapy for type 1 diabetes.

## 1. Introduction

Type 1 diabetes (T1D) remains the most common chronic immune-mediated disease in young children and adolescents. T1D is an autoimmune disorder, characterized by the destruction of the insulin-producing beta cells in the pancreas by the body's own immune system [1]. Despite a clear genetic predisposition, the initial culprit that sets the disease into motion remains unknown. The longstanding classical hypothesis proposes that activation of the immune system results in a rapid destruction of healthy pancreatic beta cells [2]. This dogma is being challenged in recent years with a

conversion to abnormal pancreatic beta cells as initial culprit [3]. This implies that a dysfunctional beta cell is cleared by a normal functioning immune system. As both roads to beta cell destruction remain plausible, the harmonizing hypothesis states that T1D is the result of a complex network of dysfunctions in both the beta cells and the immune system [4]. Nevertheless, in all hypotheses, the true quest remains to determine the initial trigger of the dysfunctional beta cells and/or immune system. Much of the etiology of T1D is accounted for by genetic predisposition. Initially, linkage studies revealed the contribution of the major histocompatibility complex (MHC) to T1D susceptibility [5]. Later, genome-wide association studies increased the number of loci known to be associated with T1D exponentially with some genes linked to beta-cell dysfunction and others to immune cell dysfunction [6]. However, the increased incidence of T1D over the past decades cannot solely be explained by genetics, but is probably related to one or more environmental factors. Of these, vitamin D is one of the most intriguing molecules, as the vitamin D receptor (VDR) has been described in the majority of immune and metabolic cell types involved in the pathogenesis of T1D [7–9]. In this chapter, the correlations between polymorphisms in the VDR or vitamin D metabolic enzymes and risk for T1D will be addressed. The main effects of vitamin D, via its activated form 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), on the beta cell, with direct implications for the pathogenesis and prevention of T1D will be described and discussed. Furthermore, the role of vitamin D and 1,25(OH)<sub>2</sub>D<sub>3</sub> in the prevention of T1D and its effects on the immune system of the animal models for the disease will be covered.



## 2. Effect of vitamin D deficiency on type 1 diabetes presentation

In humans, the association between serum levels of 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) and T1D seems unequivocal, as T1D incidence is affected both by seasonal variation (increased prevalence in children born in spring) and by latitude (higher prevalence in northern countries with less UVB radiation) [10,11]. In NOD mice, it was reported that 25(OH)D<sub>3</sub> deficiency in utero and early life increases the risk for diabetes development [12]. Also in humans, it was reported that individuals with low preclinical 25(OH)D<sub>3</sub> levels have an increased risk to develop T1D [13]. Based on these promising results, large birth cohort studies that have invited participants based on an increased genetic risk for T1D have analyzed the association between serum 25(OH)D<sub>3</sub> levels and T1D. The Finnish Diabetes Prediction and Prevention (DIPP) study [14,15], the Diabetes Auto-Immunity Study in the Young (DAISY) [16], and the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) [17] all concluded that there was no association between serum 25(OH)D<sub>3</sub> levels and risk of islet autoimmunity or T1D at birth or during childhood. The TRIGR did report that 25(OH)D<sub>3</sub> levels 18 months prior to the age of first seroconversion were lower compared with healthy controls; however, mean 25(OH)D<sub>3</sub> levels remained sufficient ( $\geq 50$  nmol/L) in all groups [17]. On the other hand, The Environmental Determinants of Diabetes in the Young (TEDDY) did report that higher mean 25(OH)D<sub>3</sub> levels in childhood and early infancy were associated with a decreased risk of islet autoimmunity [18]. Being vitamin D sufficient ( $\geq 50$  nmol/L) in childhood was associated with a 31% lower risk of islet autoimmunity compared with those that were insufficient. Vitamin D sufficiency during early infancy was associated with a 40% lower risk of islet autoimmunity compared with those who were insufficient. Recently, a dose–response metaanalysis of case–control studies examining the relation between 25(OH)D<sub>3</sub> levels and the risk of T1D reported a U-shaped association [19]. The risk of T1D descended with 25(OH)D<sub>3</sub> levels ranging from 39 to 89 nmol/L was unchanged when 25(OH)D<sub>3</sub> levels reached 103–113 nmol/L and slightly ascended when 25(OH)D<sub>3</sub> levels surpassed 150 nmol/L.

Vitamin D deficiency during pregnancy may represent a predisposing factor for the development of various autoimmune disorders, including T1D, in later life [20]. A case–control study indicated an association between lower maternal 25(OH)D<sub>3</sub> serum levels during late pregnancy and an increased risk of T1D development in the offspring [21]. However, others reported that maternal midpregnancy or neonatal cord blood 25(OH)D<sub>3</sub> levels did not have an impact on the risk of childhood T1D [15,22,23]. A metaanalysis of case–control studies examining the relation between maternal

25(OH)D<sub>3</sub> levels during pregnancy and the risk of T1D in the offspring reported that lower maternal 25(OH)D<sub>3</sub> levels during pregnancy were associated with a higher risk of T1D in the offspring [24]. Interestingly, no significant association was observed between the 25(OH)D<sub>3</sub> level during first or second gestational trimester and the risk of T1D in the offspring. Although no dose–response analysis was performed, the 25(OH)D<sub>3</sub> levels were within the sufficiency range ( $\geq 50$  nmol/L) in all groups. Not only maternal 25(OH)D<sub>3</sub> levels are potentially associated with T1D development in the offspring, but also maternal vitamin D–binding protein (DBP) levels and the maternal VDR are being investigated as potentially being associated with T1D in the offspring. Indeed, higher maternal DBP levels at delivery, but not in midpregnancy or in the child's cord blood, were associated with a lower risk of T1D in the offspring [25]. Miettinen et al. reported that maternal single-nucleotide polymorphisms (SNPs) in the VDR gene may influence the in utero environment and contribute to the early programming of T1D in the fetus [26]. It is even more complicated as the associations between serum 25(OH)D<sub>3</sub> concentration and some SNPs are stronger during pregnancy in mothers whose children later developed T1D than in mothers whose children did not [27].

Vitamin D deficiency is not only associated with the onset of T1D, but also in people with long-standing T1D, a metaanalysis of multiple observational studies revealed a higher prevalence of vitamin D deficiency compared with healthy controls [28,29].

## 3. Relation between vitamin D–related genes and the genetic predisposition to type 1 diabetes

Novel insights into a possible role of 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> in the pathogenesis of T1D originate from epidemiological data on associations between polymorphisms of the VDR, vitamin D–metabolizing enzymes *DHCR7*, *CYP2R1*, *CYP27B1*, and *CYP24A1* or the gene for DBP (*GC*), and the risk of T1D in certain ethnic communities.

1,25(OH)<sub>2</sub>D<sub>3</sub> exerts its genomic effects mainly via the nuclear VDR that heterodimerizes with retinoid X receptors and translocates into the nucleus [30] (see Chapters 11–13). VDRs are present in different tissues, including the human pancreatic beta cells and basically every cell of the immune system, and influence important physiological processes such as calcium transport as well as cell growth and differentiation [8]. The gene encoding the VDR is located in humans on chromosome 12q12–14 and has at least two promoter regions, eight protein-coding exons (namely 2–9) and six untranslated exons (1a–1f). It shows extensive SNPs including a FokI polymorphism in exon 2 (rs10735810) that alters the start

codon, BsmI, and ApaI polymorphisms in the intron between exons 8 and 9 (rs1544410 and rs7975232, respectively), a TaqI polymorphism in exon 9 (rs731236), and a polyadenylic acid mononucleotide repeat in the 3' untranslated region [31]. The ApaI and BsmI polymorphisms of the *VDR* gene are considered silent SNPs. These polymorphisms do not alter the amino acid sequence of the encoded protein but seem to be in strong linkage disequilibrium, although there are ethnic variations [32,33]. Besides these frequently studied variants, there are many other polymorphisms that are less often considered, including A1012G (A to G substitution), Cdx2 (rs11568820), and various resulting *VDR* genotype combinations. Several studies showed a correlation between some of these *VDR* polymorphisms and T1D, whereas others failed to confirm this association. A large metaanalysis found an association between BsmI polymorphism and T1D risk in an Asian population, with a 30% increased risk for carriers [34]. A study by Ban et al. revealed an association between FokI polymorphism and GAD65 positivity in a Japanese population [35]. However, the Type I Diabetes Genetics Consortium did not find any association of *VDR* SNPs with T1D in the overall sample set or in any of the subgroup analyses of the parent-of-origin, gender of offspring, and human leukocyte antigen (HLA) risk [36]. Nevertheless, vitamin D receptor response elements exist in the promotor region of at least certain HLA alleles [37]. This indicates that vitamin D metabolism and/or the genetic effects regulated via the *VDR* may be connected with the HLA-related immune response, which may have an impact on the pathogenesis of T1D [38]. This was recently confirmed by Miettinen et al. who reported that women with the HLA-B44 supertype (consisting of B\*18, B\*37, B\*40, and B\*44 alleles) had lower 25(OH)D<sub>3</sub> concentrations [38]. In addition, the FokI polymorphism of the *VDR* could have functional implications, altering ligand-mediated gene expression in beta cells or in the immune system [39]. The function of the other polymorphisms, located in introns of the *VDR*, is unknown but likely involves mRNA stability [40].

The enzymes CYP2R1 (a 25-hydroxylase [25-OHase]) and CYP27B1 (1 $\alpha$ -OHase) catalyze the metabolism of vitamin D<sub>3</sub> to 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>, the most active natural vitamin D metabolite, whereas CYP24A1 (24-OHase) is involved in the catabolism and inactivation of 1,25(OH)<sub>2</sub>D<sub>3</sub> through a series of events starting with 24-hydroxylation. Multiple studies show that polymorphisms in the *CYP2R1* gene and in the *CYP27B1* gene located on chromosome 12q13.1-q13.3 are associated with an increased risk of developing T1D [41–44]. These SNPs are assumed to reduce the (local) expression of the 1 $\alpha$ -(OH)ase enzyme and consequently the conversion of 25(OH)D<sub>3</sub> to 1,25(OH)<sub>2</sub>D<sub>3</sub>. Although genetic variants in *CYP24A1* have been shown to associate with

serum 25(OH)D<sub>3</sub> levels [45], no associations with T1D have been found. However, when SNPs at the *DHCR7*, *GC*, *CYP2R1*, and *CYP24A1* loci were combined, there was a cumulative effect on the T1D susceptibility [46]. On the other hand, a large genome-wide association study in European-ancestry individuals showed that common genetic variants are unlikely to have a strong modifying effect on increases in 25(OH)D<sub>3</sub> levels following typical dietary intakes [47]. Based on the SNPs from genes influencing 25(OH)D<sub>3</sub> levels in this study, being *CYP2R1* rs10741657, *CYP2R1* rs117913124, *DHCR7/NADSYN1* rs12785878, *GC* rs3755967, *CYP24A1* rs17216707, *AMDHD1* rs10745742, and *SEC23A* rs8018720, Hyppönen et al. performed a meta-analysis assessing associations between these variants effecting 25(OH)D<sub>3</sub> and T1D risk, revealing a null association [48].

The significance of the potential correlations between genetic contributors to vitamin D metabolism and T1D remains unclear. Nevertheless, an association of *VDR* genotypes with *VDR* mRNA as well as protein has been demonstrated in peripheral blood mononuclear cells providing functional relevance to the *VDR* polymorphisms [49]. Also, a large, nested, case–control study following 8676 children at increased genetic risk of T1D demonstrated not only that the risk of islet autoimmunity depends on the childhood 25(OH)D<sub>3</sub> level, but also that this needs be adjusted by the *VDR* genotype [18]. This was later confirmed by Stene et al. who reported that in children homozygous for the *VDR* rs11568820 G/G genotype, higher 25(OH)D<sub>3</sub> levels at birth predict a decreased risk for T1D [25]. The explanation for this observation given is that *VDR* genotype rs11568820 results in lower *VDR* expression [50].

#### 4. Vitamin D and the beta cell

Since the early observations in 1980 by Norman et al. [51] that pancreatic insulin secretion is selectively inhibited by hypovitaminosis D, several reports have demonstrated an active role for 1,25(OH)<sub>2</sub>D<sub>3</sub> in beta cell function. This is reinforced by demonstration of the presence of the *VDR* [52] in pancreatic beta cells, the expression of 1 $\alpha$ -OHase in pancreatic beta cells [53], and the presence of a vitamin D response element in the human insulin receptor gene promoter [54].

In the pathogenesis of T1D, considerable evidence indicates that the cytotoxic action of proinflammatory cytokines on pancreatic beta cells is mediated through the generation of free radicals and the induction of endoplasmic reticulum stress [55]. Due to their function in insulin synthesis and secretion, beta cells have an elaborate ER system and are thus considered to be under high ER stress. In severe ER stress, the active form of

PERK increases ATF4 expression and subsequently promotes CHOP production. CHOP is a transcription factor that induces the expression of proapoptotic factors [56].  $1,25(\text{OH})_2\text{D}_3$  is able to protect the beta cells against ER stress-associated apoptosis by inhibiting this PERK-ATF4-CHOP pathway [57]. Apoptosis results in the release of beta cell proteins, and these autoantigens can elicit an immune response. Here, DBP has been identified as a T1D autoantigen [58]. The alpha cells of the pancreatic islets are also one of the few tissues outside the liver to express significant levels of DBP [59,60]. In alpha cells, the action of intracellular DBP appears to be independent of its normal systemic cargo  $25(\text{OH})\text{D}_3$  and instead involves a well-recognized function of DBP as an actin binder (see Chapter 7). DBP-induced intracellular changes in actin have been shown to be associated with altered alpha cell morphology, leading, in turn, to modulation of beta cell function [61]. Zhang et al. later confirmed that DBP-specific epitopes elicit cytotoxic T lymphocyte responses and as such contribute to the development of T1D [62].

Inflammation plays a crucial role in T1D pathogenesis, contributing to beta cell dysfunction and apoptosis through cytokines and chemokines produced by immune cells but also by beta cells themselves [63]. In this regard,  $1,25(\text{OH})_2\text{D}_3$  and its analogs have been shown to prevent the interleukin (IL)- $1\beta$ -induced inhibition of beta cell function, as well as interferon (IFN)- $\gamma$ -stimulated beta cell expression of MHC class I and class II molecules in rat pancreatic islets [64,65]. Nevertheless, the exact mechanism by which cytokines act to promote beta cell destruction remains unclear. It has been suggested that inflammatory cytokines predispose pancreatic beta cells to lysis by autoreactive T cells.  $1,25(\text{OH})_2\text{D}_3$  does not provide direct protection against cytokine-induced beta cell death as treatment with  $1,25(\text{OH})_2\text{D}_3$  did not protect purified beta cells against cytokine-induced cell death [66]. However, Wolden-Kirk et al. showed that  $1,25(\text{OH})_2\text{D}_3$  could almost completely prevent cell death induced by inflammatory cytokines IL- $1\beta$  and IFN- $\gamma$  in human and mouse whole islets, while restoring impaired insulin secretion.

Protection of beta cells by  $1,25(\text{OH})_2\text{D}_3$  is accompanied by altered expression of genes involved in chemotaxis, cell death, and beta cell function [67]. Another observation is that  $1,25(\text{OH})_2\text{D}_3$  is not only able to alter the effect of cytokines on beta cell function, but also blocks the induction of beta cell surface markers by these cytokines. When neonatal rat islets were incubated with T helper (Th)-1 IFN- $\gamma$ , several surface markers, including those linked to antigen presentation such as MHC-II molecules, were upregulated, making them better targets for the immune system. Coincubating the islets with  $1,25(\text{OH})_2\text{D}_3$  or some of its analogs markedly

decreased the upregulation of MHC-II molecules after IFN- $\gamma$  stimulation [65]. Moreover, treatment of beta cells with  $1,25(\text{OH})_2\text{D}_3$  is able to directly protect against proinflammatory cytokine-induced beta cell death by reducing the density of MHC class I molecules [65], reducing IL-6 production and nitric oxide synthesis [68], inducing the expression of antiapoptotic A20 protein [69], and decreasing the expression of Fas [70]. Wei et al. [71] recently reported that association of the VDR with the alternative chromatin-remodeling complex PBAF enhances the VDR-dependent transcriptional program, resulting in reduced cytokine-induced beta cell proinflammatory response and preserved beta cell function in human beta-like cells and in diabetic mouse models. Complementing these findings, we observed that exposure to  $1,25(\text{OH})_2\text{D}_3$  in three cell systems (e.g., INS-1E beta cell line, FACS-purified rat beta cells, and nonobese diabetic (NOD)-severe combined immune-deficient islets) suppressed chemokine CXCL10 and IL-15 expression in the beta cell itself but did not prevent cytokine-induced beta cell death [66]. These data show beta cell protection against inflammatory agents involved in the pathogenesis of T1D and thus may have direct implications for the observed in vivo effects of  $1,25(\text{OH})_2\text{D}_3$  and its analogs in the prevention of T1D in animal models and humans. Vitamin D (metabolites) make beta cells less visible to the immune system (cell surface markers) and reduce the attraction signals for the immune system secreted by beta cells themselves.

The influence of vitamin D on beta cells was further tested in animal models of T1D such as the spontaneously diabetic NOD mouse model and the streptozotocin (STZ)-induced mouse model of T1D. A remarkable observation in the NOD mouse is that *Vdr* gene expression is decreased in islets of mice that develop diabetes [72]. Overexpression of *Vdr* is able to ameliorate diabetes in transgenic NOD mice by increasing the proliferation capacity of the beta cell and by reducing local inflammation as the antiinflammatory A20 mRNA was increased and the proinflammatory *Tnfa*, *Tgfb*, and *H2Aa* mRNA levels were decreased compared with control [72]. Moreover, vitamin D seems to have an effect on cathepsin G (CatG). CatG is the only protease involved in the antigen presentation of proinsulin, and a CatG-specific inhibitor can improve the beta cell function by a reduced activation of  $\text{CD4}^+$  T cells, thus slowing the progression of diabetes in NOD mice [73]. Lai et al. reported that vitamin D supplementation also functions as CatG inhibitor in diabetic NOD mice as it is able to improve beta cell function by a downregulation of CatG and thus inhibition of  $\text{CD4}^+$  T cell activation [74].

In the STZ mouse model, a single high dose of STZ (70–250 mg/kg body weight) causes a rapid and

complete destruction of beta cells. On the other hand, multiple lower doses of STZ (e.g., 50 mg/kg on five consecutive days) cause more subtle beta cell damage followed by insulin deficiency model [75]. In the low doses of STZ model, treatment with  $1,25(\text{OH})_2\text{D}_3$  reduced diabetes incidence from 85% to 45% [76]. In a study of Inaba et al., both the high- and the low-dose models of STZ were used to test the effect of  $1\alpha\text{-(OH)D}_3$ , a precursor of  $1,25(\text{OH})_2\text{D}_3$ , on diabetes prevention [77]. The rationale for utilizing  $1\alpha\text{-(OH)D}_3$  is that this will be hydroxylated in the liver to generate the bioactive  $1,25(\text{OH})_2\text{D}_3$  compound in vivo. In the single high-dose STZ model, no protection against diabetes was observed with  $1\alpha\text{-(OH)D}_3$ . When multiple low doses of STZ were administered,  $1\alpha\text{-(OH)D}_3$  reduced the diabetes incidence dramatically to 46% compared with 100% in control mice. Histological examination of the pancreas of these experimental mice demonstrated that  $1\alpha\text{-(OH)D}_3$  also reduced insulinitis. A major criticism of this model remains the fact that the beta cell destruction is probably the result of nonspecific inflammatory damage and not a true (auto)immune-mediated model for diabetes [78]. Indeed, disease progression is not dependent on the presence of an autoimmune response because STZ is able to induce diabetes even in the absence of functional T and B cells. Furthermore, in this STZ-induced model, it was also demonstrated that  $1,25(\text{OH})_2\text{D}_3$  injections protected beta cells against apoptosis and reversed insulinitis by autophagy [79], which is essential for the survival and function of the beta cells [80]. Here,  $1,25(\text{OH})_2\text{D}_3$  was able to enhance the expression of LC3 and Beclin 1, essential proteins involved in autophagy, and therefore accelerate the renewal of organelles under stress, together with an increased expression of the antiapoptotic protein Bcl-2 [79].

## 5. Vitamin D as an immune modulator in type 1 diabetes

The VDR is present on almost every cell of the immune system, including macrophages, dendritic cells (DCs), and activated B and T cells, allowing them to respond to the active ligand [8] (see Chapters 94–96). Therefore, a physiological role for  $1,25(\text{OH})_2\text{D}_3$  as a potential immune regulator is reasonable, and so far, many studies yielded beneficial results using active vitamin D to prevent and/or intervene in different autoimmune disease models including T1D [81,82]. Results in autoimmune diabetes-prone NOD mice are promising, but many obstacles to human application still exist. Clinical application of  $1,25(\text{OH})_2\text{D}_3$  is hindered by toxicity because the supraphysiological doses needed to shape the immune system also elicit hypercalcemic side

effects. All studies involving long-term immune suppression are inconceivable as strategy for the prevention of a chronic disease in children and adolescents. Moreover, preliminary results on the beneficial effects of these drugs in recent-onset diabetic subjects have been disappointing [83–85].

Interestingly, most of the aforementioned immune cells are able to convert  $25(\text{OH})\text{D}_3$  into  $1,25(\text{OH})_2\text{D}_3$  as they express the *CYP27B1* gene ( $1\alpha\text{-OHase}$ ) (see Chapter 9), while DCs also express a  $25\text{-OHase}$  [86,87]. Local processing of vitamin D precursors into the bioactive metabolite may represent an important mechanism by which immune cells can reach these supraphysiological levels of  $1,25(\text{OH})_2\text{D}_3$  inside the cell without negative feedback by  $1,25(\text{OH})_2\text{D}_3$  itself [86]. This mechanism results locally in high concentrations, which are needed to modulate immune responses autonomously, without changing the systemic concentrations of this hormone [88]. These intracrine, autocrine, and paracrine signaling events are critically dependent on sufficient  $25(\text{OH})\text{D}_3$  levels available to the cell as substrate for the  $1\alpha\text{-OHase}$ . This was recently illustrated by Th1 cells expressing *CYP27B1*. Here, an autocrine/paracrine loop permits Th1 cells to both activate and respond to vitamin D as part of a shutdown program repressing  $\text{IFN-}\gamma$  and enhancing IL-10 [89]. Based on these findings, supplementation with vitamin D represents an attractive strategy to overcome vitamin D deficiency and ensure adequate immune cell function. Nevertheless the effect of vitamin D on immune cells is complex as illustrated by the fact that VDR expression in immune cells (e.g., T cells or monocytes) is sometimes differently controlled according to the corresponding activation status [90,91].

## 6. Interventions using vitamin D to prevent or arrest type 1 diabetes

### 6.1 Animal studies

The NOD mouse and biobreeding (BB) rat are the two most commonly used animals that spontaneously develop diabetes, comparable with human type 1 diabetes [92]. As such are these models crucial in providing insights into genetic susceptibility and mechanisms of disease pathogenesis, but also in the translational development of novel therapies [93]. It was in fact in the NOD mouse model in which it was first demonstrated that chronic administration of pharmacological doses of  $1,25(\text{OH})_2\text{D}_3$  can reduce the incidence of both insulinitis (the histological lesion of diabetes) and diabetes [94,95].

In these prevention trials, the timing and product of the vitamin D supplements turned out to be crucial. Regardless of the dosing regimen or type of vitamin D product used, only early and long-term treatment



prevented diabetes in NOD mice [66,96]. In the life of a NOD mouse, weaning is at 3 weeks of age, and insulinitis (the immune infiltration of the islets of Langerhans, and thus histological reflection of beta cell attack) starts at 6–8 weeks of age. Diabetes onset starts from 10 weeks of age, with a peak at 14–16 weeks of age, and can occur until 30 weeks of age. Vitamin D intervention from 14 weeks of age (i.e., in the presence of insulinitis) failed to prevent disease [66]. This implies treatment must be started early. A relevant question is if long-term treatment with  $1,25(\text{OH})_2\text{D}_3$  is necessary for disease protection or if a short-term intervention would suffice. If so, when in the course of the disease should this short-term treatment be given? Vitamin D from 3 weeks of age until 14 weeks of age is able to reduce diabetes onset, but is inferior compared with a lifelong continuation of the intervention [66,96]. This is exemplified by Gysemans et al. [66] showing a reduction of diabetes from 58% in control NOD mice compared with 20% in NOD mice treated lifelong with  $1,25(\text{OH})_2\text{D}_3$  or to 35% in mice treated only until 14 weeks of age. Others also reported that vitamin D (16 IU by gavage) administered in utero (via the mother), and the first 10 weeks of life did not change the incidence of diabetes or modify the disease processes that leads to beta cell destruction in the NOD mouse [97].

Besides timing, the type of product and the dosing regimen of the vitamin D supplements are also crucial. Animal studies are essential in the exploration of the product and dosing regimen in vitamin D interventions as they bridge in vitro research with human trials. In vitro research is often an artificial situation, with continued exposure of the immune cell subsets to (often supraphysiological) high doses of vitamin D products, mostly  $1,25(\text{OH})_2\text{D}_3$  [9]. These concentrations are often not achievable in human peripheral blood but could potentially be reached at local sites of inflammation as many immune cells can produce vitamin D metabolites themselves upon activation [8,98].  $1,25(\text{OH})_2\text{D}_3$ , the active form of vitamin D, reduces the diabetes incidence in NOD mice when given either intraperitoneally at  $5\text{ }\mu\text{g/kg}$  body weight every other day [66,95] or orally via incorporation of the hormone in the diet ( $50\text{ ng/day}$ ) [99]. However, these interventions resulted in increased serum calcium levels, increased bone turnover (reflected in serum osteocalcin levels), and bone loss. A caveat in these studies is that many dietary changes can have an effect on the disease incidence as exemplified by Zella et al. [99] where the diet besides being enriched in  $1,25(\text{OH})_2\text{D}_3$  was also low calcium. This resulted in an unusually low spontaneous disease incidence in the control mice. When mice were given a normal-calcium full-grain diet,  $1,25(\text{OH})_2\text{D}_3$  treatment did not reduce diabetes incidence to previously reported levels, but control mice had a normal spontaneous disease incidence [100].

As most cells of the immune system have the appropriate machinery to process vitamin  $\text{D}_3$  into its bioactive compound, natural vitamin  $\text{D}_3$  may ultimately be more desirable for clinical use [8]. Here, in contrast to  $1,25(\text{OH})_2\text{D}_3$ , the method of administration seems to make a difference. Peritoneal injection of 1000 IU/day of solubilized vitamin  $\text{D}_3$  from 3 to 70 days of age was unable to provide protection against diabetes development in NOD mice by 30 weeks of age [101]. However, it must be taken into account that treatment was not given lifelong. In contrast, lifelong dietary supplementation with a high-dose vitamin  $\text{D}_3$  (800 IU/day) from weaning to 35 weeks of age did significantly reduce diabetes incidence with 52% (females) and 58% (males) in NOD mice [96]. No calcium toxicity was observed, while pancreatic insulin content was preserved and the insulinitis score improved.

In immune-mediated diseases, vitamin D results in a shift in the immune status toward the induction of tolerance and is as such appealing as an immune modulator in immune-mediated diseases [9]. In the NOD mouse model, the basis for interference with the immune response was found to be by restoration of the suppressor cell function [95]. Later, it was demonstrated that the nature of the suppressor cell induced by both  $1,25(\text{OH})_2\text{D}_3$  and vitamin  $\text{D}_3$  is most likely a  $\text{CD4}^+$  regulatory T cell (Treg) [96,102]. Tregs, characterized by a combination of surface markers including CD25, CD103, CTLA-4, CD62L, glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related protein, glycoprotein-A repetitions predominant, and by expression of the forkhead/winged helix transcription factor (FoxP3), have been implicated as key players in immune tolerance (see Chapter 96). The key role of Tregs in diabetes occurring in NOD mice has been confirmed by cyclophosphamide (CTX). CTX administration induces diabetes in NOD mice due to a reduction in naturally occurring Tregs [103,104]. Shortly after CTX treatment,  $\text{CD4}^+\text{CD25}^+$  Tregs are functionally impaired in their suppressive activity and display higher levels of apoptosis [103]. While cell numbers recover in lymphoid tissues immediately before onset of diabetes, frequencies of Tregs in the pancreas remain low.

Several potential preventive therapies have already been tested in this CTX model of T1D, as this model is able to determine if protection against diabetes by therapeutic interventions is only by the induction of suppressor T cells and not by autoreactive T cells [105,106]. Casteels et al. used this model and demonstrated that  $1,25(\text{OH})_2\text{D}_3$  can prevent diabetes induced by CTX in the NOD mouse [107]. Insulinitis was significantly reduced from 100% in control NOD mice to 42% in  $1,25(\text{OH})_2\text{D}_3$ -treated NOD mice, whereas diabetes itself was reduced from 78% to 17%. As such, protection was achieved despite a total elimination of

immunosuppressive cells in the  $1,25(\text{OH})_2\text{D}_3$ -treated group by CTX (as shown by cotransfer experiments).  $1,25(\text{OH})_2\text{D}_3$  treatment also did not interfere with the quantitative and qualitative recovery of the major lymphoid-hematopoietic cells after CTX administration. Striking was the absence of insulitis in most animals treated with  $1,25(\text{OH})_2\text{D}_3$ . Both the resistance against CTX and reduction of insulitis together with the absence of protection in cotransfer experiments suggested that CTX had eliminated Tregs. It appears that these suppressive cells are not the sole protective mechanism in the vitamin D-treated NOD mice. It is reported that also  $\text{CD11b}^+\text{Ly6G}^-\text{Ly6C}^+$  monocytes might contribute to the adjuvant effect of CTX, but no data are available on the effects of  $1,25(\text{OH})_2\text{D}_3$  on this myeloid cell population and its immunosuppressive capacities [108]. Furthermore, Takiishi et al. reported that vitamin  $\text{D}_3$  supplementation decreases effector  $\text{CD8}^+\text{IFN-}\gamma$  and  $\text{CD4}^+\text{IFN-}\gamma$  T cells [96].

The BB rat is another spontaneous animal model for T1D. In this model, no difference in diabetes incidence between control (20%) and  $1,25(\text{OH})_2\text{D}_3$ -treated rats (29% and 39%) was observed, while  $1,25(\text{OH})_2\text{D}_3$  (0.2 or 1  $\mu\text{g}/\text{kg}/2$  days, respectively) was administered from 3 to 50 days of age [101]. In this model, also vitamin  $\text{D}_3$  (1000 IU/day) was not able to prevent diabetes by early-life treatment (from 3 to 50 days of age) [101].

## 6.2 Human studies

Based on previous observations, the association between vitamin D and T1D seems unequivocal, and vitamin D deficiency is postulated as one of the potential triggers of T1D onset. However, even more than in animal studies, the design of human trials is hampered by uncertainty of product choice, timing of intervention, and dosing regimen [9]. It has to be taken into account that even the definition of vitamin D insufficiency and deficiency is based on the skeletal effects of vitamin D, and these definitions may not apply to levels needed for normal functioning of beta cells or the immune system (see Chapters 51 and 52) [109].

T1D is a disease affecting young children and adolescents, necessitating interventions early in life when prevention of the disease is attempted. Intervention trials in animal studies, as described earlier, have demonstrated that early and lifelong interventions can prevent T1D onset [95,96]. In humans, several trials intervened already during pregnancy by optimizing the maternal intake of vitamin D [110,111]. Here, Stene et al. demonstrated that cod liver oil taken during pregnancy can reduce T1D in offspring. Fronczak et al. as part of the DAISY recruitment trial, confirmed that

maternal intake of vitamin D through food during pregnancy was associated with a decreased risk of islet autoimmunity in offspring [112]. Not all reports come to the same conclusion, however, as Virtanen et al. did not find a correlation between maternal intake of vitamin D during pregnancy and the risk for the development of diabetes-associated autoantibodies or clinical T1D in the offspring [111]. A possible reason for this discrepancy is that the latter was a retrospective trial, which is subject to recall bias. Despite these mixed results, Sorensen et al. found a clear association between low  $25(\text{OH})\text{D}_3$  levels during pregnancy and an increased risk for T1D [21]. Here, blood samples were mainly taken in the last trimester of the pregnancy and demonstrated that children born from a mother with a  $25(\text{OH})\text{D}_3$  level of  $\leq 54$  nmol/L had a more than twofold risk of developing T1D compared with children born from a mother with a  $25(\text{OH})\text{D}_3$  level of  $> 89$  nmol/L.

Several trials reported an association between vitamin D supplementation early in life, even already starting during the first year of life, and a decreased risk for T1D [113–117]. A metaanalysis of data from four case–control studies revealed that children being supplemented with vitamin D had a 29% reduction in the risk of developing T1D compared with unsupplemented controls [116]. The optimal timing of the intervention remains unclear, but is suggested to be more beneficial from 7 to 12 months of age than supplementation from birth to 6 months of age [113]. The dosing regimen and product of supplementation remain uncertain. In the majority of these studies, no information was given about the dosing regimen or product that was used [116]. On the other hand, Hypponen et al. reported that the intake of 2000 IU vitamin  $\text{D}_3$  during the first year of life diminished the risk of developing T1D [115]. Here, the authors showed some evidence of a dose–response and regularity effect, with those using higher and more frequent amounts of vitamin D being at lower risk of developing T1D. Taking into account that T1D susceptibility is linked to specific HLA genotypes, Wicklow and Taback intended to pursue a trial using 2000 IU/day of cholecalciferol in newborn babies with increased HLA-associated risk [117]. Although the dose was found to be safe, the clinical trial addressing the effect of cholecalciferol supplementation on T1D risk in newborns has not yet been initiated. However, in a small prospective trial, 12 high-risk children, aged 1.5–13 years old, with islet cell autoantibodies were supplemented with oral calcitriol (0.25  $\mu\text{g}/\text{day}$ ) for 1–3 years. Here,  $1,25(\text{OH})_2\text{D}_3$  was able to decrease serum autoantibody levels against GAD65 and insulin in all participants, suggesting to be effective in the prevention of T1D [118].

Besides its impact as environmental trigger in T1D and thus its role in the prevention of T1D, vitamin D has meaningful immunomodulatory characteristics [9]. This warrants its use in new-onset (and potentially established) T1D to help preserve the residual beta cell function. A retrospective analysis in children and adolescents newly diagnosed with T1D indeed reported that low 25(OH)D<sub>3</sub> levels are related to a higher HbA1c at diagnosis and that if these individuals did not receive supplementation, there was a worse glycemic control versus those with baseline repletion [119]. Here, researchers face the same problems, being the uncertainty about timing, product, and dosing regimen of the vitamin D intervention. As a rule of thumb, the earlier the intervention, the more residual beta cell function is to be saved in newly diagnosed T1D. With regard to the product of intervention, clinical data on the use of 1,25(OH)<sub>2</sub>D<sub>3</sub>, starting when the decline of beta cell function is already ongoing, are disappointing. A small intervention trial, in which new-onset diabetic children were given a small dose of 1,25(OH)<sub>2</sub>D<sub>3</sub> (0.25 µg/2 days), showed that there was no improvement of C-peptide levels, although insulin requirements decreased in the 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated group [83]. An increase of the dose to 0.25 µg 1,25(OH)<sub>2</sub>D<sub>3</sub> (probably the maximum tolerable dose) was tested in two intervention trials [84,85], but both showed no protective effect on the residual beta cell function as C-peptide, fasting C-peptide, HbA1c, and insulin requirement were all comparable with the control group. Even when specified in recent-onset patients with high basal C-peptide levels, no protective effect was observed [84]. In contrast to these unsuccessful interventions with 1,25(OH)<sub>2</sub>D<sub>3</sub>, interventions with vitamin D<sub>3</sub> did show some protection. A potential reason for this is that interventions with vitamin D<sub>3</sub> allow the use of higher dosing regimens without a significant risk of hypercalcemia. Here, interventions with 2000 IU/day for 18 months [120] or 140,000 IU/month for 3 months [121] did show a slower decline of C-peptide levels compared with the control group. An intervention with 50,000 IU/week for 2 months and then 50,000 IU/biweekly for 10 months did not show differences compared with the control group after 12 months [122]. However, in the vitamin D<sub>3</sub> intervention group, there was a decreased rate of increase in both HbA1c and insulin dose-adjusted HbA1c.

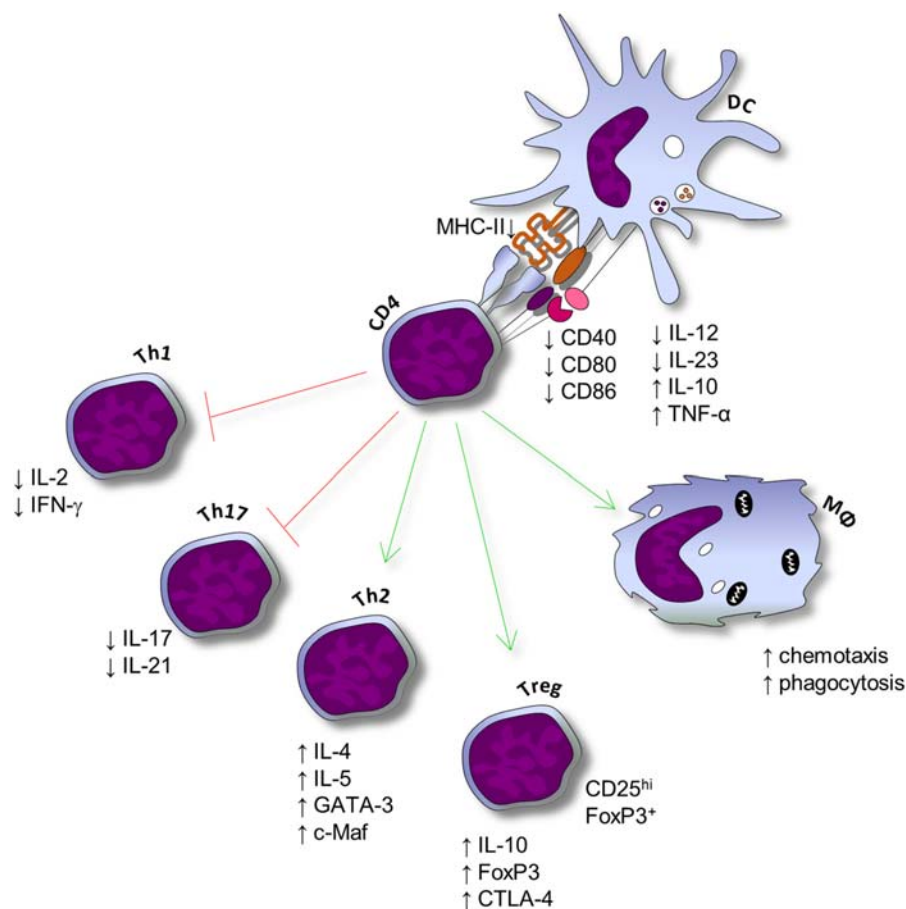
To avoid enrollment during the honeymoon period, when C-peptide concentrations may remain variable regardless of the intervention, Panjiyar et al. decided to intervene in T1D patients 1–2 years postdiagnosis with 3000 IU/day vitamin D<sub>3</sub> for 1 year [123]. In the vitamin D<sub>3</sub> intervention group, there was a decrease in fasting blood glucose level, HbA1c, and stimulated C-peptide compared with the control group.

Moving further along the timeline of disease progression, also in people with long-standing T1D, the impact of optimizing vitamin D levels on the glucometabolic control has been explored. Interventions in long-standing T1D with 4000 IU/day [124], 10,000 IU/day [125], or 50,000 IU biweekly [126] vitamin D<sub>3</sub> resulted in an improved glucometabolic control with a reduction in HbA1c, insulin demands, fasting blood sugar levels, lower glycemic variability, and a lower frequency of hypoglycemia. Despite promising data, not all trials come to the same conclusion. Some did not find an improvement in glucometabolic control by interventions with 4000 IU/day [127], 10,000 IU/day [127], or monthly doses of 60,000 IU to 120,000 IU vitamin D<sub>3</sub> [128]. Nevertheless, vitamin D-insufficient patients with long-standing T1D had a similar therapeutic response to single dose of 150,000 IU vitamin D<sub>3</sub> as healthy controls with similar baseline 25(OH)D<sub>3</sub> levels [129]. This suggests that insulin deficiency alone does not significantly affect either the regulation of vitamin D metabolism or the activity of the enzymes involved. It is possible that the immunomodulatory effects of vitamin D<sub>3</sub> only occur at optimal serum concentrations maintained for a sufficiently long period to achieve protection on the residual beta cell function. This is a plausible hypothesis, explaining why certain studies using bolus doses of vitamin D<sub>3</sub> do not produce appreciable benefits on residual beta cell function [123].

Finally, vitamin D supplementation has been explored in latent autoimmune diabetes in adults (LADA). This is a subtype of T1D in which the clinical manifestations begin in adulthood and progress slowly. Patients with LADA who received 1α-(OH)D<sub>3</sub>, the synthetic precursor of 1,25(OH)<sub>2</sub>D<sub>3</sub>, exhibited a partial preservation of beta cell function in comparison with patients treated with insulin alone [130].

## 7. Immune effects of interventions with vitamin D in type 1 diabetes

At present, the proposed basis of protection by interventions with vitamin D and its metabolites seems to be a reshaping of the immune repertoire next to protective beta cell effects (Fig. 100.1). In general, vitamin D induces a more tolerogenic immune environment. Its regulatory actions involve a shift in T cell cytokine profiles from predominantly proinflammatory Th1 (e.g., IL-2, IFN-γ, and TNF-α) and Th17 (e.g., IL-17) to antiinflammatory Th2 (e.g., IL-4, IL-10) profiles [102,131]. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> directly promotes the generation of IL-10-producing Tregs [132,133]. This was later confirmed in both animal [96] and human [134] trials. These Tregs were found to have an enhanced suppressive capacity as defined by the reduction of effector



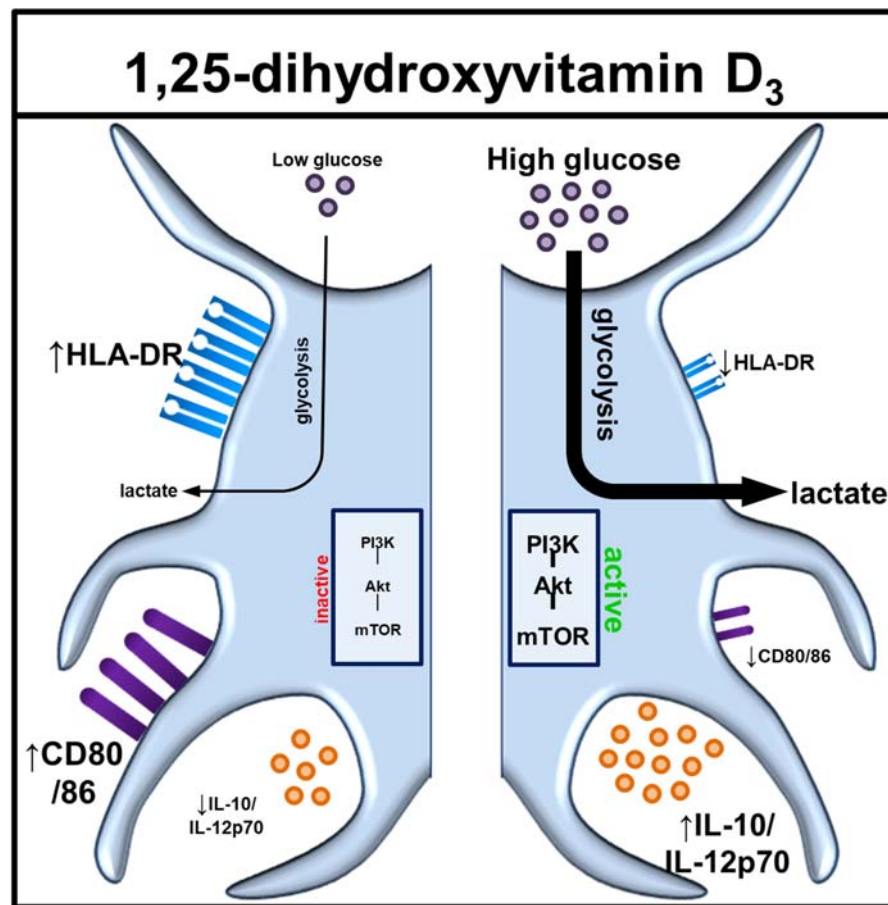
**FIGURE 100.1** At the level of the DC, 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the surface expression of MHC-II and of costimulatory molecules as well as the production of the cytokine IL-12 (and IL-23), together with a stimulation of IL-10 and TNF-α, thereby indirectly shifting the polarization of T cells from a Th1 toward a Th2 phenotype. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> acts as immune modulator directly at the level of the T cell by inhibiting the production of the Th1 cytokines IL-2 and IFN-γ (red arrows), Th17 cytokine IL-17 while stimulating the production of Th2 cytokines such as IL-4, IL-5, and transcription factors GATA-3 and c-MAF (green arrows). Moreover, 1,25(OH)<sub>2</sub>D<sub>3</sub> favors the induction of regulatory T cells (Tregs). 1,25(OH)<sub>2</sub>D<sub>3</sub> also improves chemotactic and phagocytic capacity of macrophages. Adapted from [8].

T cells proliferation [121]. In a study comparing T1D subsets with their healthy siblings, Savastio et al. reported in the healthy individuals that low levels of 25(OH)D<sub>3</sub> allow expansion of Th17 cells that may be counteracted by increased Treg/ICOS<sup>+</sup> levels by vitamin D supplementation [135]. This was particularly seen in healthy siblings carrying protective haplotypes as they showed the highest levels of Treg/ICOS<sup>+</sup> cells without substantial differences in Th17 after vitamin D supplementation, while T1D subsets did not display this expansion of Treg/ICOS<sup>+</sup> cells. This suggests that vitamin D is unable to act when the disease is already established in T1D, indicating that supplementation could be more useful in the prevention phase before the beta cell destruction.

In addition to its direct actions on T cells [90], 1,25(OH)<sub>2</sub>D<sub>3</sub> can also modulate T cell responses indirectly via effects on antigen-presenting DC [136,137]. Indeed, 1,25(OH)<sub>2</sub>D<sub>3</sub> is able to reshape DCs toward

tolerogenic cells with reduced expression of antigen-presenting and costimulatory molecules as well as proinflammatory cytokines, thereby affecting the DC-T cell stimulatory capacity resulting in T cell anergy and the induction of antigen-specific Tregs [138–144]. In human monocyte-derived DCs, induction of the tolerogenic profile by 1,25(OH)<sub>2</sub>D<sub>3</sub> is accompanied by an early metabolic reprogramming [145,146]. This metabolic reprogramming is unique for 1,25(OH)<sub>2</sub>D<sub>3</sub> and favors oxidative phosphorylation, which is accompanied by increased aerobic glycolysis. The result is that 1,25(OH)<sub>2</sub>D<sub>3</sub>-conditioned fully differentiated DCs, but not tolerogenic DCs induced by other biological and pharmacological agents, rely on glucose availability and usage, as well as on the PI3K/Akt/mTOR pathway, for the induction and maintenance of their tolerogenic phenotype [146] (Fig. 100.2). The glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4) is a critical checkpoint in this process as





**FIGURE 100.2** 1,25(OH)<sub>2</sub>D<sub>3</sub> reshapes DCs toward a more tolerogenic profile by the activation of the glucose metabolism in these cells. This is unique for 1,25(OH)<sub>2</sub>D<sub>3</sub> and implies that 1,25(OH)<sub>2</sub>D<sub>3</sub>-conditioned fully differentiated DC, but not tolerogenic DCs induced by other biological and pharmacological agents, rely on glucose availability and glycolysis controlled by the PI3K/Akt/mTOR pathway, for the induction and maintenance of their tolerogenic phenotype and function. The glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4) is a critical checkpoint in this process. *Original figure from [146].*

inhibition of this enzyme blocks 1,25(OH)<sub>2</sub>D<sub>3</sub>-conditioned DCs' capacity to induce suppressive Tregs [147]. Epigenetic modifications further contribute to the stability of tolerogenic DCs, as they are able to persist perturbation by inflammatory stimuli [148,149]. Although these two studies found that about a third of the transcripts encoded by non-HLA T1D were differentially between inflammatory DCs and 1,25(OH)<sub>2</sub>D<sub>3</sub>-conditioned DCs, only five of these genes were direct targets of the VDR. The understanding these genetic risk loci holds the potential to develop specific or selective disease intervention strategies [150]. In vivo, it has been demonstrated that DCs generated in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub> can redirect already committed T cell clones derived from a T1D patient toward nonproliferation [140]. In the NOD mouse, reshaping of the immune system happens centrally, in the thymus, where treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> restores the sensitivity of T lymphocytes toward apoptosis-inducing signals, allowing only "activation-induced cell death"—sensitive T cells

to reach the periphery [107,151,152]. In vivo transfer of islet antigen-loaded 1,25(OH)<sub>2</sub>D<sub>3</sub>-modulated DCs could successfully prevent islet allograft rejection [144]. Moreover, their in vivo migratory capacity remained intact and favored homing to the pancreas of adult NOD mice [143].

## 8. Vitamin D analogs and type 1 diabetes

A major obstacle to the human application of 1,25(OH)<sub>2</sub>D<sub>3</sub> is its adverse effects on calcium and bone metabolism. New structural analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub> have been reported, displaying a clear dissociation of calcemic and antiproliferative effects [153,154]. Some chemical modifications of the side chain and A-ring results in "superanalogs" with 10–100-fold more activity on cell differentiation and the immune system than native 1,25(OH)<sub>2</sub>D<sub>3</sub>. Nonsteroidal analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub>, lacking either the full five-membered D-

ring (C-ring analogs) or the full six-membered C-ring (D-ring analogs), are designed and documented to be very effective inhibitors of cell proliferation or inducers of cell differentiation than is  $1,25(\text{OH})_2\text{D}_3$  [154,155]. Interestingly, several of the vitamin D analogs possess more potent immunomodulatory properties, resulting in protection against autoimmunity and prolongation of allograft survival [136,156,157]. Some of the most promising analogs, coming from different chemical laboratories, have been tested in the NOD mouse [156,158] as well as in the BB rat [159]. Also in humans, the use of analogs in T1D is promising as a small clinical trial with alfacalcidol (0.25  $\mu\text{g}$  twice daily) treatment in newly diagnosed children showed preservation of beta cell function [160]. The mechanism of protection against insulinitis and diabetes appears to be similar to that of mother compound  $1,25(\text{OH})_2\text{D}_3$ . Exposure to vitamin D analogs enhances both chemotactic and phagocytotic capacity of monocytes/macrophages, while their antigen-presenting cell function decreases [161]. In DCs, analogs have an effect on cell morphology, cell surface marker, and cytokine expression (e.g., MHC-II, CD80, CD86, IL-12, and IL-10), and endocytic and migratory capacity. Hereby,  $1,25(\text{OH})_2\text{D}_3$  analogs significantly alter the antigen-presenting function of DC [172] both in vitro and in vivo, resulting in suppression of T cell activation [8,162–164]. Moreover, effects of analogs on the induction of Tregs [90,132,133], the redirection of T cells to sites of inflammation [132], and beta cell protection have been described [8,165]. In the search for the optimal analog, a combination of beta cell protection, immune modulation, and low calcemic effects is desirable.

## 9. Vitamin D in combination therapy to prevent or arrest type 1 diabetes

The hope that T1D would one day be cured by one drug or intervention is long forgotten. T1D is always described as being one entity as the clinical endpoint of insulin deficiency, requiring lifelong exogenous insulin substitution, remains the same. However, T1D is actually a collective term for a heterogeneous disease [166]. Secondly, as described before, the true culprit of disease initiation, either the pancreatic beta cell and/or an aberrant immune system, remains to be determined [166]. These observations make us realize that the solution might lay in combination therapy.

In animal models of T1D, disease prevention can be achieved by the chronic use of a myriad of different drugs such as  $1,25(\text{OH})_2\text{D}_3$ , azathioprine, cyclosporine, tacrolimus, nicotinamide, and anti-CD3, whereas disease intervention is only (partially) possible by some of these agents, such as anti-CD3 [167]. The development

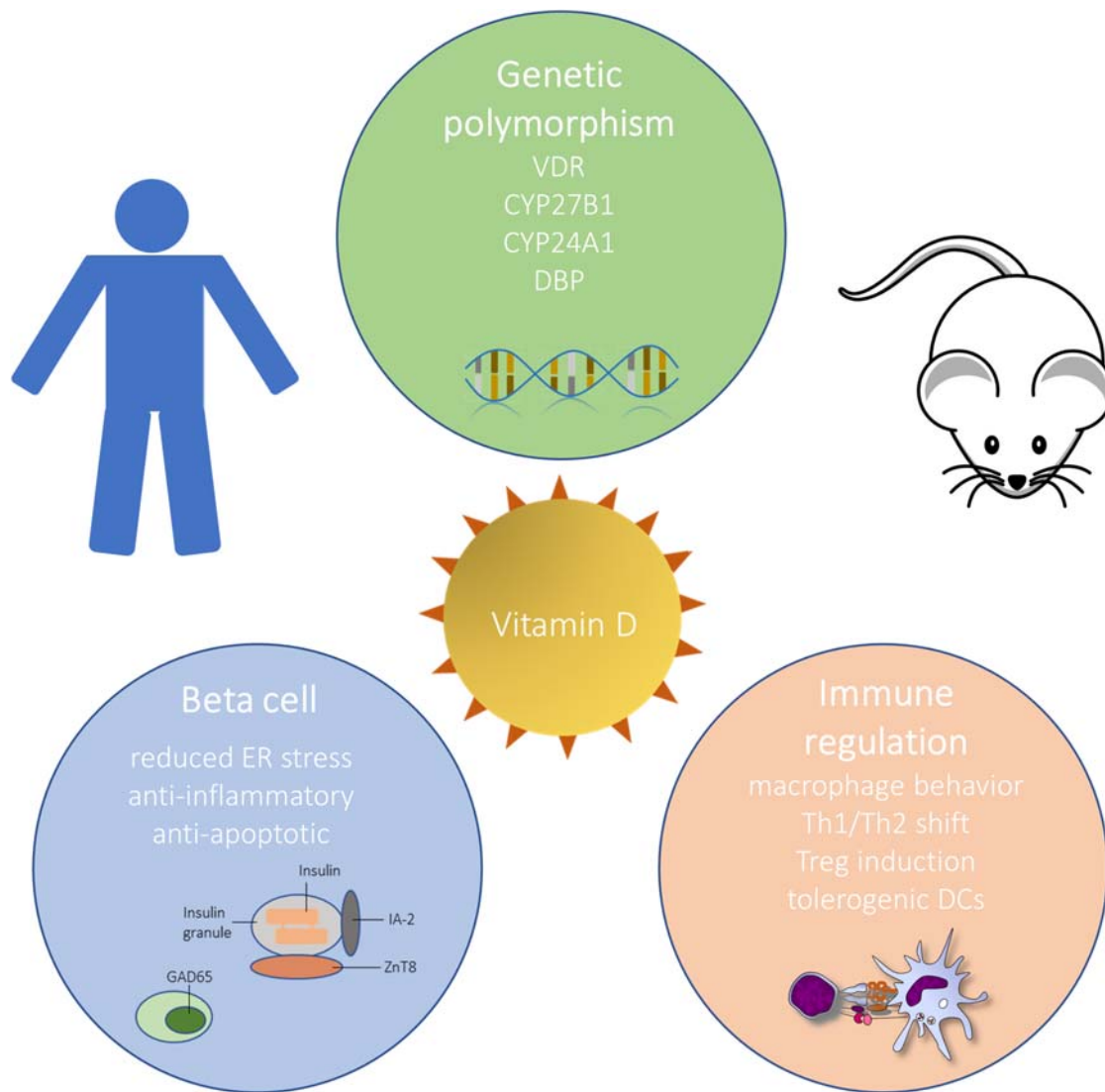
of drugs that are highly selective and yet produce minimal toxicity to the host remains one of the most complicated challenges in (autoimmune) disease protection. Moreover, because the majority of diseases are treated with drugs in combination regimens rather than single agents, one practical approach to circumvent toxicity is to develop combination regimens that will potentiate the effectiveness of current (clinical) protocols without its toxicity. This strategy would accelerate the acceptance of new drugs as adjunct strategies because these molecules could be used at concentrations well below their maximal tolerable doses. For example, azathioprine can cause pancreatitis and bone marrow suppression, which may increase the risk of infection or serious bleeding [168]. Common adverse effects of calcineurin inhibitors such as cyclosporine and tacrolimus are bowel disturbance, nephrotoxicity, neurotoxicity, and serious side effects on pancreatic beta cell function such as a reduced insulin synthesis and beta cell toxicity [169,170]. Several combinations of vitamin D analogs with other immune modulators have been tested both in vitro and in vivo. Five candidates have been tested for a combination therapy with  $1,25(\text{OH})_2\text{D}_3$  (cyclosporine, tacrolimus, rapamycin, leflunomide, and mycophenolate mofetil [MMF]) and showed maximum synergism with cyclosporine [171,172]. Other immunomodulators such as type I IFNs that exhibit a broader range of immunomodulatory properties (mainly restriction of T cell proliferation and IFN- $\gamma$  production in part by inhibiting DC-mediated IL-12 secretion while promoting IL-10 production) also gave interesting results in combination with less hypercalcemic vitamin D analogs in experimental models of multiple sclerosis and T1D [173,174].

In NOD mice, diabetes can be prevented by vitamin D metabolites and analogs when treatment is started before insulinitis is present [156]. A critical question for the applicability of these analogs in the human situation is, if the vitamin D analogs can arrest progression to overt diabetes, which is the situation in prediabetic subjects in whom immune intervention is considered. Castells et al. demonstrated that some of these analogs when combined with a short induction course of cyclosporine can arrest the progression of the disease when administered after autoimmunity has already started [175]. The mechanism of protection in our model was clearly neither a generalized immune suppression nor the induction of different immune cell subsets, and cotransfer experiments with splenocytes from animals treated with the combination therapy did not reveal the presence of suppressor cells. Signs of local immunoregulation were, however, prominent locally in the islets. The approach of utilizing vitamin D analogs at concentrations that produce limited hypercalcemia, as adjuncts to conventional immunotherapy, is very

promising and might open new perspectives in the protection against T1D in humans.

In comparison with other immunosuppressive agents, vitamin D is an appealing addition in combination with antigen-based therapy due to its unique profile. Most immunosuppressive agents target T cells, or some, such as MMF, both T and B cells. Conversely, no immunomodulatory agent in clinical use specifically targets antigen-presenting cells and in particular DCs, which are known to be involved in T cell activation and tolerance induction. Vitamin D, however, is able to inhibit DC differentiation and maturation into APCs, therefore rendering DCs more tolerogenic [138–144]. The importance of these tolerogenic DCs generated from monocytes by a combined treatment with vitamin D and antigen-specific therapy is that they induce antigen-specific Tregs [176]. This feature is being exploited in antigen-based immunotherapy. Roep et al. demonstrated that proinsulin loaded vitamin D<sub>3</sub>-treated and dexamethasone-treated tolerogenic DCs in humans are safe [177]. Although further trials are needed, the first results suggest a delay or halt of the beta cell destruction. To improve efficacy of subcutaneous injections with glutamic acid decarboxylase (GAD) together with aluminum hydroxide (alum) as carrier, also Ludvigsson et al. decided to add 2000 IU/day vitamin D<sub>3</sub> to the treatment regimen [178]. Here, the addition of vitamin D<sub>3</sub> resulted in a tendency to a less rapid decline of C-peptide from 6 to 30 months with increasing serum 25(OH)D<sub>3</sub> levels being positively correlated with C-peptide preservation [178]. These trials are well underway as Ludvigsson et al. decided in a follow-up trial to change the mode of injection to intralymphatic administrations of GAD-alum, again together with 2000 IU/d vitamin D<sub>3</sub> supplementation [179]. The addition of vitamin D was again hypothesized to improve the efficacy of the treatment by its effect on the immune system [180]. In this trial, the preservation of C-peptide was only seen in patients carrying HLA DR3-DQ2 [179]. This was later confirmed in a follow-up trial specifically in T1D patients with HLA DR3-DQ2 compared with random T1D patients [181]. Based on 25(OH)D<sub>3</sub> levels, it seemed unlikely that the observed efficacy was conferred by the vitamin D supplementation [179]. This tolerogenic influence on DCs by vitamin D is also being exploited in specific bioengineering approaches to safely deliver effective antigen-based vaccines to intervene in T1D. A combination microparticle system has been established, with encapsulated vitamin D<sub>3</sub>, TGF-β1, GM-CSF, and the insulin B(9–23) peptide, which is able to modulate DC phenotype in vivo. Two subcutaneous injections with these particles could reduce diabetes incidence and insulinitis in female NOD mice, while Treg frequency increased [182,183].

Lastly, vitamin D is being exploited in combination with islet or stem cell transplantation in T1D. In T1D, the insulin-producing beta cells in pancreatic islets are destroyed by autoreactive T cells. The long-lasting persistence of autoreactive T cells in human blood and the memory phenotype displayed by at least some of these immune cells suggest the existence of an autoimmune memory [184]. The latter phenomenon is responsible for the destruction of MHC-matched or syngeneic beta cells, transplanted under the form of isolated beta cells, islets, or whole pancreas [185]. Successful transplantation requires the prevention of allograft rejection and the break of the autoimmune memory. Some analogs of 1,25(OH)<sub>2</sub>D<sub>3</sub> have been tested for their capacity to prevent disease recurrence after islet transplantation in spontaneously diabetic NOD mice. The most spectacular results were obtained with a combination of KH1060 (20-epi-22-oxa-24,26,27-trishomo-1,25(OH)<sub>2</sub>D<sub>3</sub>) and subtherapeutic doses of cyclosporine [186]. In the group receiving KH1060 (0.5 µg/kg/2 days) together with cyclosporine (7.5 mg/kg/day), a synergistic effect between both drugs was observed: four out of seven mice maintained a functioning graft for 60 days and did not show recurrence for at least 30 days after stopping the treatment. Treatment was administered from the day before transplantation until diabetes recurrence or in case of persistent normoglycemia until 60 days after transplantation. The subtherapeutic doses of KH1060 together with cyclosporine were nontoxic and had minor effects on serum calcium levels, although osteocalcin levels were clearly elevated, and bone calcium content was decreased. In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> improved islet graft survival time in diabetic rats through inhibition of nonspecific inflammation [187]. Another approach can be found in combinations of vitamin D analogs and other natural immune modulators. For example, subtherapeutic doses of recombinant (r) IFN-β alone (1 × 10<sup>5</sup> IU/day) had minor effects on autoimmune diabetes recurrence after islet transplantation (20.8 ± 14.2 days vs. 10.8 ± 2.9 days in controls). However, interestingly, a combination of rIFN-β with TX527 (14-epi-19-nor-20-epi-23-yne-1,25(OH)<sub>2</sub>D<sub>3</sub>) maintained islet graft function in 100% of mice during treatment and resulted in a marked delay of autoimmune diabetes recurrence (61.6 ± 19.6 days) [174]. Moreover, the combination of rIFN-β with TX527 resulted in an inhibition of the Th1 pathway (IL-12, IL-2, and IFN-γ), which is known to be associated with the pathogenesis of organ-specific autoimmune diseases. In addition, enhanced expression of the regulatory cytokine, IL-10, by rIFN-β and TX527 therapy, was observed. A study by Baeke et al. reported that combining low doses of anti-CD3, TX527, and cyclosporine can protect NOD mice from diabetes recurrence after syngeneic islet



**FIGURE 100.3** The pathogenesis of T1D is a complex interplay of genetic predisposition, the pancreatic beta cell, and the immune system, resulting in the autoimmune destruction of the insulin-producing beta cell. Vitamin D and its metabolite  $1,25(\text{OH})_2\text{D}_3$  have the ability to intervene at all three levels, thereby affecting disease onset and progression. Genetic polymorphisms in vitamin D–related genes (e.g., VDR, DBP,  $1\alpha$ -OHase (CYP27B1)) and  $24\text{-OHase}$  (CYP24A1) have been shown to associate with an increased diabetes risk. In addition, vitamin D deficiency due to a lack of sunlight exposure, low vitamin D dietary intake, or a defect in vitamin D–metabolizing enzymes is a risk factor influencing disease onset. In this context, vitamin D supplementation during the first year of life could reduce T1D in NOD mice and humans. The presence of the VDR in the pancreatic beta cells, the expression of  $1\alpha$ -hydroxylase in pancreatic beta cells, and the presence of a vitamin D response element in the human insulin receptor gene promoter indicate that vitamin D has an influence on the beta cell. Indeed, beta cell function is improved by  $1,25(\text{OH})_2\text{D}_3$  as it protects against ER-induced beta cell stress. Vitamin D almost completely prevents beta cell death induced by inflammatory cytokines  $\text{IL-1}\beta$  and  $\text{IFN-}\gamma$  as it makes them less visible for the immune system and reduces the attraction signals for the immune system secreted by beta cells themselves. Furthermore, vitamin D induces the expression of antiapoptotic A20 protein. The presence of the VDR as well as vitamin D–metabolizing enzymes in several cells from the immune system was a first indication for the immunomodulatory effects of  $1,25(\text{OH})_2\text{D}_3$ . In macrophages,  $1,25(\text{OH})_2\text{D}_3$  increases the chemotactic and phagocytic capacity together with enhanced antimicrobial properties. In addition,  $1,25(\text{OH})_2\text{D}_3$  induces a shift from Th1 and Th17 toward a Th2 T cell response. Moreover, T cell behavior is also indirectly regulated by  $1,25(\text{OH})_2\text{D}_3$  through modulation of dendritic cell (DC) phenotype. Here,  $1,25(\text{OH})_2\text{D}_3$  is able to reshape DCs toward tolerogenic cells with reduced expression of antigen-presenting and costimulatory molecules as well as proinflammatory cytokines, subsequently affecting its T cell stimulatory capacity resulting in T cell anergy and induction of antigen-specific Tregs. As a result of the multilevel impact of vitamin D, this molecule could have a contributing role for the prevention or treatment of T1D; however, solid clinical data is currently lacking.

transplantation [188]. Remarkably, mice receiving all three drugs survived longer ( $69 \pm 10$  days) than mice receiving one or two agents and remained normoglycemic during the whole treatment period. Combining

these drugs enhances their individual potency but also offers an interesting strategy to circumvent dose-related side effects of immunosuppressants currently used in clinical transplantation.



More recently, stem cell transplantation has emerged as a potential treatment for T1D due to its intrinsic regenerative capacity and immunomodulatory properties, which may arrest the autoimmune beta cell destruction and generate functional beta cells [189]. Allogenic adipose tissue–derived stromal/stem cells are tested together with vitamin D as immunosuppressant in patients with recent onset T1D [190]. This resulted in stability of C-peptide, better glucose control, and lower insulin requirements compared with controls. A larger prospective open trial is currently ongoing ([ClinicalTrials.gov](https://clinicaltrials.gov) no. NCT03920397).

## 10. Conclusion

Since the discovery that sunlight affects the onset of T1D with increased incidence rates in the winter months and in regions further away from the equator, the relation between vitamin D and T1D has been indisputable. Whereas the initial hypothesis was that vitamin D deficiency would be an environmental factor that could potentially trigger the onset of T1D, extensive research in the past four decades demonstrated an indisputable relation between vitamin D and all the different players in the pathogenesis of T1D. Both in vitro and in vivo trials in both animal models and humans demonstrated that the key aspect of vitamin D is in the prevention of T1D. This is achieved by interacting with both the beta cells and the immune system (Fig. 100.3). Recent research demonstrates an even more complex and elaborate association. In particular, T1D starts in a genetically at-risk individual where polymorphisms of the *VDR*, the vitamin D-metabolizing enzymes *CYP27B1* and *CYP24A1*, or the *DBP* gene can influence the risk of T1D. Besides its crucial role in the prevention of T1D, the immunomodulatory effects of vitamin D have been explored in the stabilization of the beta cell destruction in T1D. Here, just as for other immunomodulators, clinical trials have mostly failed. An important reason might be that the choice of the vitamin D metabolite and its dose and frequency of administrations are critical factors that need to be considered when designing clinical trials. To achieve a higher dosing regimen without eliciting toxic side effects, vitamin D analogs have been designed with mixed results. Lastly, the hope that T1D could ever be prevented by one drug or intervention is long forgotten. However, due to its unique characteristics, vitamin D is an appealing add-on in combination regimens, already showing promising results.

At present, the only solid conclusion from the data on vitamin D and T1D is that vitamin D deficiency should be avoided at all cost. Long-term intervention studies are needed to demonstrate if vitamin D plays a crucial

role in the rising incidence of T1D and how and when supplementing individuals can prevent or arrest T1D.

## 11. Summary points

- There is a clear association between vitamin D deficiency and type 1 diabetes.
- Vitamin D and its analogs are able to protect the beta cell and drive the immune system toward immune tolerance.
- In animal models of T1D, high doses of vitamin D metabolites and analogs prevent type 1 diabetes.
- In humans, the role of vitamin D in preventing or arresting type 1 diabetes is unclear.
- Due to its unique immune characteristics, vitamin D is an appealing add-on in combination regimens in future trials to prevent or arrest type 1 diabetes.

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# Vitamin D mechanisms of protection in multiple sclerosis

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## OBJECTIVES

- Present foundational information on MS pathogenesis and sexual dimorphisms that characterize this autoimmune demyelinating disease.
- Review current knowledge gleaned from Mendelian randomization studies that now confirm vitamin D insufficiency as a cause for MS, and clarify how the vitamin D hormone and the nuclear VDR are likely to directly and indirectly influence MS-relevant gene expression at every step in the transcriptional process and in many types of cells.
- Explain how the MS sexual dimorphism data implicate participation of the vitamin D hormone and the nuclear VDR in a CD4<sup>+</sup> T cell intrinsic mechanism that is regulated by the sex hormones, estradiol and testosterone, and suggest a hypothetical model for hormonal regulation of the *FOXP3* gene that may explain the MS sexual dimorphism data.
- Clarify how the proinflammatory, IFN $\gamma$ -producing CD4<sup>+</sup> Th1 cells direct the immune response to pathogens, how the antiinflammatory, IL-10-producing CD4<sup>+</sup> Tr1 cells protect tissues from immune-mediated damage, and how the

vitamin D hormone and the nuclear VDR serve as a highly localized signal to drive CD4<sup>+</sup> Th1 cells to transdifferentiate into protective CD4<sup>+</sup> Tr1 cells when a pathogen has been cleared.

- Describe the importance of oligodendrocytes and the myelin sheath to proper brain function, summarize evidence that the vitamin D hormone and the nuclear VDR provide signals that promote oligodendrocyte precursor cell differentiation into myelinating oligodendrocytes, and suggest a hypothetical model for VDR regulation of the methionine cycle and epigenetic silencing of repressive genes whose function is to maintain oligodendrocyte precursor cells in an immature state.
- Suggest research challenges from the vitamin D and MS perspective to discover MS-relevant genes that are differentially expressed in the vitamin D—replete and vitamin D—deficient states and elucidate the transcriptional control mechanisms; to probe the biochemistry of vitamin D hormone synthesis in the context of inflammation and evaluate the relationship between vitamin D status and methionine cycle function; to define how the vitamin D system impacts regulatory T cells of all types and their immune checkpoints; to investigate the

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connections between nutrition, methionine cycle function, and MS; and finally, to conduct additional robust and thoughtfully designed vitamin D<sub>3</sub> intervention trials, with longitudinal measurement of biomarkers and clinical data, in an effort to find accessible indicators of a prodromal period for MS disease when a vitamin D<sub>3</sub> intervention might alter the trajectory toward an MS diagnosis.

## 1. Introduction

The first probable case of multiple sclerosis (MS), an autoimmune demyelinating disease of the central nervous system (CNS), was described in a letter written in 1757 by Patrick Brydone to Scottish physician Robert Whytt [1]. At that time, Brydone's use of "upward of 600 severe shocks" as a therapeutic trial elicited published comment from Benjamin Franklin, who had also attempted electric shock therapy in paralytic patients. Now, 265 years later, MS afflicts ~2.8 million people worldwide, with a new case being diagnosed in an adult at a highly productive stage of life about every 5 min [2]. The global MS disease burden nearly tripled in the half-century before 2013 [3,4] and has nearly doubled since then [2]. Neurological deficits gradually deprive MS patients of movement, sensory perception, cognition, emotional well-being, independence, and livelihood. Despite decades if not centuries of research and an arsenal of approved medications, MS remains incurable.

Multiple sclerosis is so named for its signature pathological features: focal demyelinating lesions disseminating in time and space [5]. The earliest focal lesions surrounding the perivascularity exhibit myelin instability and fragmentation, phagocytosis of fragments by myelin-laden macrophages, loss of myelinating capacity by oligodendrocytes (OLG), activated microglia, T cells (T lymphocytes), reactive astrocytes, and axonal loss. The focal nature of early lesions suggests that demyelination may result from a highly localized inflammatory response and possible oxidative damage to the myelin rendering it unstable and easily fragmented. The chronic lesions that develop later in the disease process share many features with the early lesions. In addition, slowly expanding lesions may be seen with smoldering, chronic inflammation, and tissue sclerosis. The neurological deficits that characterize MS are attributed to chronically demyelinated axons, consequent transmission failure, axonal degeneration, and finally, neuronal cell death.

When this chapter was last updated, deep skepticism greeted several aspects of the vitamin D-MS hypothesis [6]. Scientists debated whether the protective

association between sunlight exposure and lower risk of MS reflected cutaneous vitamin D<sub>3</sub> synthesis and the biological actions of the vitamin D hormone, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), and its vitamin D receptor (VDR). Moreover, disbelief confronted emerging data showing that relapsing-remitting MS (RRMS) incidence and prevalence had tripled in just a few decades in a female-biased manner [7]. The global scope, magnitude, and rapidity of this female-biased increase implied that it had nongenetic etiological roots. The hypothesis that the globally declining population vitamin D status might be contributing to the rapidly increasing MS incidence had not taken hold [8]. Controversy persisted concerning the proposal that extrarenal 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and autocrine and paracrine signaling within and between the immune system and the brain might influence MS risk, since these mechanisms had not been demonstrated in vivo [9]. The 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated transcriptional control of many MS-relevant genes had been demonstrated in T lymphocytes and monocytes, but how such control was sometimes asserted in the absence of defined vitamin D-responsive elements (VDREs) remained enigmatic [10].

Here, we summarize the remarkable recent advances that have deepened our understanding of the vitamin D system's protective mechanisms and strengthened the inference that vitamin D insufficiency contributes causally to MS pathogenesis, consistent with the Bradford-Hill paradigm [11]. New genetic studies have highlighted the role of epigenetic mechanisms as integration points for gene-environment interactions in MS [12]. Recent Mendelian randomization studies (reviewed in the following and in Chapter 61), and animal modeling experiments [13], have left no doubt that 25-hydroxyvitamin D<sub>3</sub> (25(OH)D) and the capacity of immune cells to produce and use 1,25(OH)<sub>2</sub>D<sub>3</sub> for autocrine and paracrine signaling strongly influence MS disease risk and severity. New immunological studies have provided more precise mechanistic details concerning the proposed CD4<sup>+</sup> T lymphocyte transdifferentiation model, wherein the presence or absence of pathogens determines highly localized 1,25(OH)<sub>2</sub>D<sub>3</sub> production and subsequent switching of proinflammatory CD4<sup>+</sup> T helper type-17 (Th17) cells into CD4<sup>+</sup>FOXP3<sup>+</sup> T regulatory (Treg) cells, as well as switching of proinflammatory CD4<sup>+</sup> T helper type-1 (Th1) cells into antiinflammatory CD4<sup>+</sup> T regulatory type-1 (Tr1) cells through the complement receptor (CD46) [14] (see Chapter 96).

Our understanding of mechanisms by which 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDR assert transcriptional control over MS-relevant genes has also deepened. Important details have emerged concerning mechanisms of 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDR transcriptional control of the *Foxp3*, *Ikzf2*, and *Ctla4* genes in CD4<sup>+</sup>FOXP3<sup>+</sup> Treg cells.

Other recent biochemical data have demonstrated 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDR transcriptional control of betaine–homocysteine methyltransferase (BHMT), the methionine (MET) cycle, and DNA-methylation in CD4<sup>+</sup> T lymphocytes [15]. The MET cycle is dysregulated in MS patients [16]. Recent research localized BHMT to the oligodendrocyte nucleus where it was bound to DNA-methyltransferase-3a (DNMT3a) [17]. Enhanced methyl donor availability in the nucleus promoted epigenetic DNA and histone methylation changes that stimulated oligodendrocyte precursor cell (OPC) maturation to a myelinating phenotype.

In this chapter, we integrate the many remarkable advances to describe how extrarenal 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and autocrine and paracrine signaling to VDR-expressing cells (see Chapter 9) determine the differentiation and function of CNS cells and immune system cells. Some mechanisms involve direct gene regulation through ligand-activated VDR interactions with gene promoters, enhancers, and other transcriptional factors. Other mechanisms require stimulation of the MET cycle to provide single carbon units for epigenetic control of cell differentiation and function. These mechanisms are exquisitely sensitive to the female sex hormone, estradiol (E2), and to the presence of pathogens. We close the chapter with a summary of the many remarkable advances, and a perspective on questions raised but not yet answered. We hope this review will stimulate new research into vitamin D mechanisms of protection in MS with the dual goals of reversing the troubling female-biased trend of increasing MS incidence and prevalence and preventing MS or reducing the impact of MS for individuals with this diagnosis.

## 2. Vitamin D and MS genetics

### 2.1 Early genetic support for vitamin D insufficiency in MS pathogenesis

A role for vitamin D insufficiency in MS pathogenesis was first suggested by the striking inverse correlation between birth latitude and MS risk in a cohort of US veterans [18], with MS being more prevalent in northern counties. Though similar MS prevalence gradients had been reported previously [19,20], this study used place of birth to control for migration and highlighted the inverse correlation with solar radiation, particularly in winter months. Latitudinal risk gradients are now confirmed in diverse populations and correlate strongly with ultraviolet radiation exposure [21] (see Chapter 102).

Migration studies suggested that MS risk associated with higher latitudes was established in early life. Risk of MS among migrants from the United Kingdom to

South Africa was reduced when migration occurred before 15–16 years of age [22]. Conversely, South Asian migrants to the United Kingdom had higher MS risk if they migrated before 15 years of age than if they migrated later [23]. Similar age-dependent alterations in MS risk have been observed in several migrant populations [24–27]. Childhood outdoor leisure activities and sun exposure were associated with lower MS risk [28–31], further supporting a role for vitamin D in early life.

A month-of-birth effect, observed in several high-latitude MS cohorts [32], provided further evidence for a seasonal risk factor. An excess of MS patients born in May following a period of relative maternal vitamin D insufficiency, together with fewer born in November, suggested that a seasonal risk factor may act in utero or in the immediate postpartum period, possibly through vitamin D–insufficient breast milk. While such studies may be confounded by year or place of birth [33], consistent observation in independent populations [34] lends credence to a seasonal factor operative in early life.

The vitamin D–MS hypothesis is highly favored to explain these observations [35], but they could in principle reflect another, as yet undefined environmental factor that varies with latitude, season, and/or sunlight exposure. Several genetic approaches have been used to test the vitamin D–MS hypothesis. In the first of these, vitamin D metabolism-related candidate genes were investigated [36]; if vitamin D insufficiency is the causal factor responsible for latitudinal gradients and seasonal effects, then genetic variation in vitamin D metabolism–related genes would be expected to influence MS risk. Early candidate gene studies provided variable support for variants near *VDR* [37–39], *CYP27B1* [36,40], and *MC1R* [39]. These early studies were underpowered, did not employ modern quality control techniques, and did not address population stratification. It is not surprising, then, that reported associations were frequently refuted in larger cohorts [36,41–44].

The advent of genome-wide association study (GWAS) methods permitted more definitive investigation of vitamin D pathways. Genes involved in vitamin D metabolism have been associated with MS in multiple studies. The *CYP24A1* gene encoding the 1,25-dihydroxyvitamin D<sub>3</sub> 24-hydroxylase (24-hydroxylase) that inactivates 1,25(OH)<sub>2</sub>D<sub>3</sub> has been consistently highlighted in several large GWAS of MS [45–47]. The *CYP27B1* gene encoding the rate-limiting enzyme for 1,25(OH)<sub>2</sub>D<sub>3</sub> biosynthesis, 25-hydroxyvitamin D 1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase), was highlighted in an early GWAS [48], replicated in an independent cohort [49] and again in meta-analysis [45]. The *CYP27B1* gene that encodes 1 $\alpha$ -hydroxylase emerged as a leading functional

candidate because this enzyme converts 25(OH)D<sub>3</sub> to active 1,25(OH)<sub>2</sub>D<sub>3</sub>. Although *CYP27B1* was an appealing functional candidate, the association lay in a gene-dense region. A subsequent fine-mapping study favored nearby *TSM* [46], whereas the most recent GWAS and meta-analysis favored *OS9* [47]. These discrepancies illustrate a major challenge in GWAS interpretation; translation of association signal to functional mechanism is difficult, and ranking genes by proximity to the lead variant is unreliable. Functional validation of GWAS results is essential, particularly in pathologically relevant tissues and environmental contexts.

Because GWAS are powered to detect common variation, they can miss rare variants (i.e., present at <1% frequency) that may have large individual effects on disease risk. Rare loss-of-function variants in *CYP27B1* may provide an example of this phenomenon. In homozygous form, several of these variants cause vitamin D hydroxylation-deficient rickets type 1A (VDDR1A; OMIM 264700) (see Chapter 66). Reasoning that VDDR1A might be a risk factor for MS, Norwegian investigators found three rare patients from two families with a co-occurrence of VDDR1A and MS, who were diagnosed with VDDR1 as children and later developed MS [50]. Applying whole-exome sequencing to a collection of multiplex MS families, investigators found suggestive association with a coding variant R389H previously known to cause VDDR1A in homozygous form [51]. This allele was not detected in a more recent, larger, international case-control study of low-frequency variants [52]. These complexities demonstrate the importance of study design and the inherent challenge of studying rare variants.

Taken together, genetic association studies provide important clues that vitamin D metabolism may influence MS risk, but these studies must be interpreted with caution. While GWAS can rigorously demonstrate association, translation of these associations into functional mechanisms is a challenging, and largely unsolved, problem.

## 2.2 Mendelian randomization studies establish vitamin D insufficiency as causal for MS

Observational epidemiology provided several lines of evidence for an inverse association between serum 25(OH)D levels and MS risk (see Chapter 102). Causal inference from observational studies is challenging: associations may be confounded by unknown or unmeasured environmental factors, measurement of potential risk factors is subject to recall bias, and risk factors may also be influenced by the disease, which is termed reverse causation. It is difficult, therefore, to translate associations between latitude or vitamin D status and MS

disease into causal statements based on observational techniques alone.

Mendelian randomization studies have been used to circumvent these issues and probe the causal relationship between low serum 25(OH)D and MS with greater certainty (see Chapter 61). In Mendelian randomization, genetic variants that determine a potentially causal factor, for example, low serum 25(OH)D levels, are identified (e.g., through GWAS, Chapter 60). The genetic variants are then used as a proxy for the factor and tested for association with a phenotype like MS, subject to a limited set of assumptions [53]. The genetic variants being used as a proxy are randomized at meiosis, whereas the disease of interest, MS, develops later in life. Thus, this causal inference framework is akin to a randomized controlled trial. Importantly, Mendelian randomization studies minimize reverse causation that has plagued other designs. In other words, the development of MS in adulthood does not alter the genetic variants that drive serum 25(OH)D levels. Consequently, Mendelian randomization studies now provide evidence of a causal relationship between the factor, low serum 25(OH)D levels, and the phenotype, MS. However, lifelong exposure to vitamin D insufficiency alone is not enough to cause MS disease; other genetic, environmental, and hormonal factors also play a role.

Genome-wide association [54–58] and whole-genome sequencing [59] studies have defined genetic factors that influence serum 25(OH)D levels (see Chapter 60). The initial SUNLIGHT GWAS identified three single-nucleotide polymorphisms (SNPs), rs6013897, rs10741657, and rs12785878, which were linked to three vitamin D metabolism genes, *CYP24A1*, *CYP2R1*, and *DHCR7*, respectively, all of which were independently associated with lower serum 25(OH)D levels [54]. There are now 143 independent SNPs that have been independently associated with serum 25(OH)D levels [58].

Using the three SNPs from the initial SUNLIGHT GWAS, Mendelian randomization demonstrated that low serum 25(OH)D level is a causal influence in both adult [60,61] and pediatric [62] MS risk. In all three studies, risk of MS increased with decreasing, genetically predicted serum 25(OH)D levels. These observations have since been replicated using larger GWASs [63]. It is important to recognize that the genetic factors employed in Mendelian randomization are present throughout the life of the individual, and thus may not adequately model vitamin D status specific to a critical period (e.g., adolescence). This minor limitation aside, genetic studies now provide strong evidence for vitamin D insufficiency as a causal influence in MS.

Mendelian randomization may also be used to disentangle multiple correlated traits. Obesity has been causally linked to MS by Mendelian randomization [64]. Since obesity may lower the bioavailability of fat-



soluble 25(OH)D<sub>3</sub>, obesity had been hypothesized to act by influencing bioavailability of vitamin D<sub>3</sub> and 25(OH)D<sub>3</sub> [65]. Using Mendelian randomization mediation analysis, both vitamin D and obesity were shown to be independently causal, with only 5.2% of the body mass index causal effect attributed to the secondary lowering of 25(OH)D<sub>3</sub> [66]. This is consistent with prior studies that did not model mediation effects but showed the causal effect of genetically determined 25(OH)D<sub>3</sub> levels to be independent of childhood body mass index [62,67].

### 2.3 Vitamin D provides epigenetic insights into MS pathogenesis

As in other autoimmune diseases, the genetic variants that drive MS risk are almost exclusively noncoding and enriched in open chromatin regions that are active in immune cells [68–70]. As a class, causal variants act by disrupting gene regulatory elements and altering gene expression. Identification of the downstream transcriptional mechanisms that transduce MS risk remains incomplete, with a minority of loci tied definitively to transcriptional targets [71]. This *variant-to-function problem* remains a major focus of the international MS genetics community. To define molecular mechanisms relevant to MS pathogenesis, we must identify, for each GWAS locus, (1) the relevant regulatory element that is disrupted, (2) the transcription factor whose binding is perturbed, (3) the gene(s) that are transcriptionally regulated, and (4) any relevant downstream molecular pathways. Epigenetic mechanisms such as histone modification and DNA methylation [72] are likely important mechanisms. A recent GWAS showed that 19 MS-associated SNPs were also associated with DNA methylation quantitative trait loci in MS patient CD4<sup>+</sup> T cells [73], providing possible insight into their mechanisms of action. To identify these downstream mechanisms for all MS loci is an enormous task, especially since chromatin accessibility, transcription factor binding, and other epigenetic mechanisms vary in different cell types and environmental conditions. We must therefore also specify the relevant cell type, and conditions under which dysregulation occurs.

The vitamin D system transduces signals derived from cutaneous exposure to the sun's ultraviolet radiation into a biologically active hormone, 1,25(OH)<sub>2</sub>D<sub>3</sub> (also termed calcitriol), that ultimately plays important roles at several points in transcriptional control of gene expression. After ingestion or production in the skin, vitamin D<sub>3</sub> is hydroxylated in the liver to 25(OH)D<sub>3</sub>, the most abundant circulating vitamin D metabolite and the best biomarker of vitamin D status. The second hydroxylation reaction is catalyzed by the 1 $\alpha$ -hydroxylase that is encoded by the *CYP27B1* gene discussed

before (see Chapters 4, 8, and 9). In the kidney and some extrarenal tissues [74], the 1 $\alpha$ -hydroxylase produces 1,25(OH)<sub>2</sub>D<sub>3</sub> (see Chapters 8 and 9). The 1,25(OH)<sub>2</sub>D<sub>3</sub> then binds to the VDR-retinoid X receptor heterodimer. This ligand-activated heterodimer binds to genomic VDREs where it influences transcription of a diverse array of genes (see Chapters 10–12).

The specific transcriptional relevance of the liganded VDR in MS was suggested by discovery of a putative VDRE in the promoter region of the *HLA-DRB* gene [75]. Excluding the rare loss-of-function *CYP27B1* mutations mentioned before, *HLA-DRB1\*1501* is the strongest genetic determinant of MS risk, with an odds ratio between 2 and 3 [76]. The putative VDRE sequence was most strongly conserved on *HLA-DRB1\*15* haplotypes, varying on other human leukocyte antigen (HLA) haplotypes with diverse effects on MS risk. In transfection experiments using lymphoblastoid cell lines, *HLA-DRB1* expression was induced upon stimulation with 1,25(OH)<sub>2</sub>D<sub>3</sub>. However, appropriate biochemical experiments such as systematic mutational analyses, VDR-RXR binding experiments, and promoter activation studies were not performed to test the function of this putative VDRE sequence in vitro or in vivo in primary B cells (see Chapter 13). Although the B lymphoblastoid cell line studies focused attention on a critical MS risk allele, they have not yet provided biochemical evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling enhances its expression by means of a VDRE.

Outside the HLA region, VDREs have been mapped genome-wide using the chromatin immunoprecipitation and sequencing (ChIP-seq) method. In this technique, cellular DNA is cross-linked to bound proteins, sheared into short fragments, and then immunoprecipitated using antibodies against a protein of interest (e.g., VDR). Sequencing of DNA fragments bound to the protein of interest provides a genome-wide distribution of its binding sites. Initial studies investigated VDR binding in B lymphoblastoid cell lines [77] or the THP-1 monocytic leukemia cell line [78,79], both before and after treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub>. VDR binding increased dramatically in both systems after 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulation. In B lymphoblastoid cell lines, the VDR bound to 623 sites at baseline, increasing to 2776 after 36 h of stimulation [77]. The VDR bound to several MS-associated loci discovered by GWAS, including *IRF8* and *PTPN2*. In THP-1 monocytic leukemia cells, 1820 VDR-binding sites were identified after 40 min of stimulation [78], with 11,657 reaching nominal significance over a 24 h time course [79]. Both studies identified numerous genes differentially expressed upon 1,25(OH)<sub>2</sub>D<sub>3</sub> stimulation [77,79,80], with many of these associated with transient or persistent VDR binding [80] (see Chapters 12 and 13).

Disease-relevant transcriptional targets of the ligand-activated VDR are best identified in disease-relevant



cells, because chromatin accessibility and transcription factor binding vary across cell types. Following the demonstration that  $1,25(\text{OH})_2\text{D}_3$  action directly on the VDR in  $\text{CD4}^+$  T lymphocytes was necessary and sufficient for experimental autoimmune encephalomyelitis (EAE) inhibition in rodents [81], investigators mapped the ligand-activated VDR binding sites in primary human  $\text{CD4}^+$  T cells using the ChIP-seq method [82]. The number of VDR-bound sites in the  $\text{CD4}^+$  T cell DNA increased with the serum  $25(\text{OH})\text{D}$  level of the cell donor, and samples from donors with higher  $25(\text{OH})\text{D}$  levels exhibited a higher proportion of intronic binding. The VDR binding sites overlapped with previously identified ChIP-seq peaks for VDR cofactors SP1, ETS1, NR4A1, and c-MYC, with CTCF-binding sites in K562 chronic myelogenous leukemia cells, and with chromatin accessibility in  $\text{CD4}^+$  Th1 cells. The VDR binding was enriched near SNPs associated with autoimmune disease, confirming the importance of the ligand-activated VDR to the transcriptional mechanisms of autoimmune disease. These findings argue for additional, larger studies in more diverse collections of primary human immune cells.

A comprehensive DNA methylation analysis of peripheral blood monocytes from MS cases and healthy controls identified a differentially methylated region (DMR) encompassing *HLA-DRB1* exon 2, with lower overall methylation in the MS cases [83]. In homozygous carriers of the *DRB1\*1501* risk gene, this DMR was significantly hypomethylated and predominantly expressed compared with heterozygous carriers and noncarriers. Locus-specific pyrosequencing confirmed the hypomethylation status of homozygous *DRB1\*1501* carriers. Causal inference analysis and Mendelian randomization revealed a significant causal relationship between methylation at DMR3 or DMR4 and reduced *HLA-DRB1* gene expression in monocytes. Lastly, these investigators identified an MS-associated variant that was undetectable by conventional genetic analysis and appeared to protect against MS by modulating DNA methylation in *HLA-DRB1*. It will be very interesting to learn whether an individual's vitamin D status is associated with the methylation status of these DMRs in *HLA-DRB1* exon 2. In particular, it would be most interesting to examine this question in the context of monozygotic twins who are discordant for MS disease, as was done previously in a longitudinal study of monozygotic twins who were discordant for type 1 diabetes to discover a DMR that preceded disease diagnosis [84].

Since the transcriptional effects of ligand-activated VDR signaling are likely to vary in different cell types, recent studies have begun to examine discrete immune cell subsets sorted by multiparameter flow cytometry. Profiling a set of 570 immune-related genes and microRNAs in flow-sorted primary human T cells

demonstrated that the *GM-CSF* transcripts were reduced and the *IL10* transcripts were increased in the memory  $\text{CD4}^+$  T cell subset after stimulation with anti-CD3 and anti-CD28 in the presence of  $1,25(\text{OH})_2\text{D}_3$  [85]. The  $1,25(\text{OH})_2\text{D}_3$ –VDR signaling also induced immunomodulatory cytokine genes *IL10* and *IL6*, along with several miRNAs and transcription factors such as *RUNX1*. Focusing on MS-associated genes in  $\text{CD4}^+$  T cells, results showed that *IL2RA* was induced, and *TAGAP* was repressed, on in vitro treatment with  $1,25(\text{OH})_2\text{D}_3$  [86]. These studies illustrate the need for larger transcriptional studies in specific primary human immune cell subsets.

In addition to its direct influence on gene transcription, VDR signaling is likely to influence MS-relevant gene expression through a variety of indirect epigenetic mechanisms. The ligand-activated VDR appears to act as a pioneer transcription factor, opening chromatin for subsequent gene regulation either directly [80] or by recruiting other pioneer factors [87,88] (see Chapter 12). The ligand-activated VDR may alter topologically associating domains and histone modifications [89] (see Chapter 13). Thus, the ligand-activated VDR is involved at every step in the epigenetic regulation of gene expression, from chromatin opening and recruitment of pioneer factors through direct action as a transcriptional regulator.

### 3. MS sexual dimorphism

#### 3.1 Female–male differences provide insight into MS etiology

Understanding the vitamin D system's role in MS molecular etiology will require mechanistic knowledge of its contributions to the sexual dimorphisms that characterize this disease. The highly significant female–male differences in MS prevalence, incidence, disease natural history, onset age, effects of puberty, and risk gene penetrance are not understood [90–93]. Most concerning is the rising global F:M MS incidence ratio attributed to an increase in female cases [7]. Between the 1930 and 1989 birth cohorts, the composite F:M MS incidence ratio increased from 1.9 to 4.6 in high-latitude countries ( $>45$  degrees), and from 1.5 to 2.3 in low-latitude countries ( $<45$  degrees) [94]. Over the same time period, population vitamin D status has declined precipitously [7]. The latitude gradient and the coincidence of the MS and vitamin D status trends suggest a causal relationship. The consistent correlation between latitude, decreasing sunlight exposure, decreasing  $25(\text{OH})\text{D}$  levels, and increasing MS prevalence has recently strengthened [95–97]. Taken together, these observations raise the question what biochemical mechanisms

could link declining 25(OH)D status with increasing MS risk in a female-biased manner?

The correlation between increasing 25(OH)D levels, increasing CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, and decreasing CD4<sup>+</sup> Th17 cells is a consistent observation in humans and mice, but the genetic and biochemical basis for it is unknown. The previous version of this chapter addressed this question through a detailed discussion of the synergy between 1,25(OH)<sub>2</sub>D<sub>3</sub> and 17β-estradiol (E2) in CD4<sup>+</sup> T cells that yielded more numerous CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and fewer CD4<sup>+</sup> Th17 cells [6]. Loss of this synergy with declining 25(OH)D levels in females but not males could be a mechanism linking vitamin D status to MS risk in a female-biased manner.

In this updated chapter, we summarize and interpret MS sexual dimorphisms to derive insight into the underlying mechanism(s). The sex hormones E2 and testosterone (TST) and epigenetic control of sex chromosome-linked genes such as the human *FOXP3* and rodent *Foxp* genes are strongly implicated in these mechanisms. Accordingly, we next summarize and interpret observations concerning the impact of 1,25(OH)<sub>2</sub>D<sub>3</sub> and the sex hormones on CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. Thereafter, we review transcriptional control of the *FOXP3* gene in some detail to derive a hypothetical model for hormonal regulation of its transcription. We close with this hypothetical model wherein the VDR, estrogen receptor-α (ERα), and androgen receptor (AR) engage with individual *FOXP3* gene enhancer regions in CD4<sup>+</sup> T cells to activate the gene promoter differently in females and males.

### 3.2 F:M incidence data over time and in transsexual individuals suggests hormone–gene interactions influence female MS risk

The most significant sexual dimorphism in MS is the threefold higher disease risk faced by women compared with men (Table 101.1). This sexual dimorphism is commonly attributed to the stronger female innate and adaptive immune response to pathogens, which is characteristic of all vertebrate species [109]. An example is the stronger female immune response to pathogen-associated molecular patterns, which has been attributed to XX versus XY chromosomal differences in the expression of a pattern recognition receptor [92]. Another example is the stronger female interferon-α response to viral infections. This sex difference has been attributed to E2 and ERα enhancement of *IFNA1* gene transcription [110]. These mechanisms are important, and there is little doubt that sex hormones and sex chromosomes contribute to them. However,

these commonly cited mechanisms rely on fixed traits, specifically, the female versus male differences in XX versus XY DNA sequences and/or sex hormones. Mechanisms based solely on fixed traits cannot explain temporal trends. The temporal trend in female MS risk calls for a mechanism that includes a corresponding temporal trend in the suspected driver. The upward temporal trend in population hypovitaminosis D incidence is the best candidate for the driver of rising female MS incidence.

Sex differences exist with respect to MS risk heritability within multiplex families. In particular, there is a strong maternal parent-of-origin effect. In half-siblings, MS recurrence risk is highest when the MS index case is the mother [111]; in aunt/uncle–niece/nephew pairs [112], risk is highest when the index case is the aunt. In a large cohort of MS multiplex families, the F:M case ratio was higher for individuals possessing the *HLA-DRB1\*15* allele than for those who were *HLA-DRB1\*15*-negative [100]. Expression of this HLA class II allele may be regulated by 1,25(OH)<sub>2</sub>D<sub>3</sub> [75]. Together, these observations suggest a model wherein interactions involving 1,25(OH)<sub>2</sub>D<sub>3</sub> and the sex hormones influence the expression of an MS risk gene, for example, *HLA-DRB1\*15*, by an epigenetic mechanism that exhibits inheritance, decay, and female gender bias.

The impact of puberty on MS incidence also demonstrates sexual dimorphism. A seminal study noted an F:M MS incidence ratio of 3.7 after puberty compared with 1.6 before puberty [101]. Additional reports confirmed a two- to threefold increase in the F:M MS incidence ratio after puberty due to increased MS incidence in postpubertal females. Duquette et al. noted “this change at female puberty is so highly significant that it must be related ... to the acquisition of the disease,” and further, “a possible endocrine explanation for the unbalanced sex ratio ... probably acting on T lymphocyte subsets” is implicated. With male puberty, TST increases from 0.2 to ~20 nmol/L, and E2 increases from 0.003 to ~0.05 nmol/L; with female puberty, E2 increases from <0.002 to ~0.4 nmol/L (peak level), and TST increases from 0.2 to 0.8 nmol/L [113]. These data suggest estrus-level E2 increases MS disease risk, whereas rising TST levels have little impact on male MS risk.

Hormone treatment for gender incongruence influenced the subsequent risk of MS differently in transsexual females and males in a small pioneering study performed in the United Kingdom. Unexpectedly, the MS risk was 4.6-fold higher in transsexual females than transsexual males [105]. Treatment for the male → female transition involves administering estrus-level E2 plus an androgen antagonist [114]. Treatment for the female → male transition involves administering TST plus progesterone [115,116]. This small pioneering

**TABLE 101.1** Sexual dimorphism in MS.

Observation	Interpretation	References
The global F:M disease prevalence ratio has risen from 1.4 to >3 due to a rise in female RRMS incidence	Fixed traits such as sex chromosome and sex hormone differences alone cannot explain this trend; instead, an environmental risk factor appears to be acting by a female-biased epigenetic mechanism	[2,6]
MS risk gene penetrance is higher in female than male MS family members	These data implicate sex hormone–gene interactions influencing risk gene expression by a female-biased epigenetic mechanism	[98–100]
The F:M disease incidence ratio is two- to fivefold higher after puberty than before puberty due to a rise in female RRMS incidence	This observation implicates estrous E2 levels ( $\sim 0.4\text{--}0.7\text{ nmol/L}$ serum) in a mechanism that increases female MS risk	[101–104]
Hormonal treatment for gender incongruence resulted in a 4.6-fold higher MS risk for transsexual females than transsexual males	Sex chromosome DNA differences are excluded, and E2 therapy for the male to female transition is unambiguously implicated in a mechanism that increases transsexual female MS risk	[105]
Pregnancy-induced hormonal changes decreased the MS relapse rate $\sim 70\%$ ; postpartum, this rate rebounded to $\sim 170\%$ of the prepregnancy year	Pregnancy-induced hormonal changes are implicated in a mechanism that supports female reproductive success and promotes peripheral immune self-tolerance	[106–108]

study implicates estrus-level E2 in a biological mechanism that increases transsexual female MS disease risk.

Further research is needed on the impact of hormone treatment for gender incongruence as it relates to the subsequent risk of MS. This unique situation constitutes a de facto treatment study in humans, where the effect of the sex hormones is observed independently of the sex chromosomes. In follow-up studies, it will be important to monitor circulating 25(OH)D levels, since population levels tend to vary with ambient solar radiation, and 25(OH)D variation would be expected to influence MS risk.

### 3.3 Pregnancy studies suggest a hormonally triggered reversible mechanism supporting reproductive success triggers MS remissions

Pregnancy studies have yielded additional insights into the relationship between hormones and RRMS disease. The 70% decline in MS relapse rates during pregnancy and the rebound to 170% of the prepregnancy year postpartum is a consistent observation [106]. Pregnancy-induced hormonal changes include a  $\sim 200$ -fold increase in E2 to a peak of  $\sim 75\text{ nmol/L}$  serum. It is not widely appreciated that 1,25(OH) $_2$ D $_3$  also

increases, specifically from 112 pmol/L prepregnancy to 260 pmol/L during pregnancy [117]. These observations suggest a model wherein pregnancy-level E2 and/or 1,25(OH)<sub>2</sub>D<sub>3</sub> participate in a reversible, protective biological mechanism related to reproductive success. This reversibility criterion excludes sex hormone impacts on thymic T lymphocyte development in childhood (but not adulthood) [118]. Pregnancy-level E2 has a well-established role in the expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells to maintain maternal tolerance to paternal antigens expressed on the fetus [119]. A question remains as to why pregnancy E2 and/or 1,25(OH)<sub>2</sub>D<sub>3</sub> levels correlate with decreased MS disease activity, whereas estrus-level E2 appears to amplify female MS risk. Risk mechanisms and disease activity mechanisms may not fully overlap; this may be one example of a divergence in the risk and disease mechanisms. Alternatively, there may be a core protective mechanism like CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell expansion that can be triggered by pregnancy-level E2 but not estrus-level E2.

In summary, reviewing data on MS sexual dimorphisms and pregnancy suggests the following key features of a biological mechanism tied to sexual disparities in MS. The involvement of 1,25(OH)<sub>2</sub>D<sub>3</sub> in a protective mechanism is suggested by the inverse correlation between two global trends, rising F:M MS incidence ratios and declining population vitamin D status. The likelihood that interactions between 1,25(OH)<sub>2</sub>D<sub>3</sub> and the sex hormones influence risk gene expression by an epigenetic mechanism is suggested by the *HLA-DRB1\*15*-positive MS multiplex family studies. A strong link between estrus-level E2 and elevated female MS risk is implied by the puberty data and the de facto sex hormone treatment study for gender incongruence. Finally, the concept that the protective biological mechanism is reversible, involves pregnancy-level E2 and/or 1,25(OH)<sub>2</sub>D<sub>3</sub>, and leads to the expansion of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells for reproductive success is suggested by the pregnancy data.

Studies in the EAE model of MS have probed for biological mechanisms that may be involved in MS sexual dimorphisms. These studies were reviewed and illustrated previously [6]. Briefly, ER $\alpha$  and VDR expression in CD4<sup>+</sup> T lymphocytes were necessary for E2 and 1,25(OH)<sub>2</sub>D<sub>3</sub>, respectively, to inhibit EAE induction. Surprisingly, E2-mediated EAE resistance required a functional *Vdr* gene in the CD4<sup>+</sup> T cells [120]. The E2 levels were higher in wild-type mice than in mice with CD4<sup>+</sup> T cells lacking a functional *Vdr* gene, suggesting these T cells either produced E2 or stimulated its production. The E2 decreased *Cyp24a1* transcripts and increased *Vdr* transcripts in the CD4<sup>+</sup> T cells, prolonging the 1,25(OH)<sub>2</sub>D<sub>3</sub> half-life and increasing CD4<sup>+</sup> T cell responsiveness to this hormone. The synergistic E2 and 1,25(OH)<sub>2</sub>D<sub>3</sub> interactions yielded an increased

frequency of CD4<sup>+</sup>Foxp3<sup>+</sup>Helios<sup>+</sup> Treg cells, higher expression of the Foxp3 and Helios proteins, and improved EAE resistance. The EAE data are consistent with a model wherein E2 and 1,25(OH)<sub>2</sub>D<sub>3</sub> interact with *Foxp3* and *Ikzf2* gene enhancer regions to increase transcription by epigenetic mechanisms. Subsequently, the Foxp3 and Helios proteins re-program the activated CD4<sup>+</sup> T cell transcriptome. Finally, the increased frequency and function of CD4<sup>+</sup>Foxp3<sup>+</sup>Helios<sup>+</sup> Treg cells improves the animal's resistance to EAE.

#### 4. CD4<sup>+</sup>FOXP3<sup>+</sup> T regulatory cells and hormonal regulation of FOXP3

##### 4.1 Sexual dimorphisms implicate a hormonally regulated, CD4<sup>+</sup> T cell intrinsic protective mechanism

The MS sexual dimorphism studies and animal modeling experiments suggest there exists a CD4<sup>+</sup> T cell intrinsic mechanism involving 1,25(OH)<sub>2</sub>D<sub>3</sub>, E2, and TST as positive transcriptional regulators of a gene or genes that is/are necessary to prevent autoimmune-mediated damage to the CNS. The hypothesized mechanism would also have an essential function in female reproduction. The human CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and *FOXP3* and rodent *Foxp3* genes fulfill these criteria. These genes establish the transcriptional identity and function of the CD4<sup>+</sup>Foxp3<sup>+</sup> T cells that prevent autoimmune-mediated CNS damage [121]. Their expression is reversibly regulated by the nuclear steroid hormone receptors VDR [81,122,123], AR [124–126], and ER $\alpha$  [127–130] through epigenetic mechanisms. Finally, selective CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell migration to the fetomaternal interface and replenishment of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells from the thymus are essential for successful pregnancy [131–133]. In the present section, we briefly review the discovery of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and the *Foxp3* gene. We then summarize the CD4<sup>+</sup> T cell heterogeneity and phenotypic malleability, in particular, the influence of 1,25(OH)<sub>2</sub>D<sub>3</sub> and the VDR on the transdifferentiation of CD4<sup>+</sup> Th17 cells into CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells that limit CD4<sup>+</sup> Th17 cell pathogenicity.

##### 4.2 The discovery of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and their dysfunction in MS

The discovery of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells can be traced to experiments probing the role of the thymus in immunological self-tolerance. Early neonatal thymectomy caused autoimmune oophoritis in female mice but not orchitis in males, implying neonatal thymus-dependent enforcement of immune privilege in the ovaries but not in the testes [134]. Transferring a minor



subset of CD4<sup>+</sup>CD25<sup>+</sup> T cells into the athymic females restored ovarian immune privilege and prevented oophoritis [135]. Human studies subsequently confirmed the pathogenic role of CD4<sup>+</sup>CD25<sup>+</sup> T cells in autoimmune disease and the dominant protective role of CD4<sup>+</sup>CD25<sup>+</sup> T cells in autoimmune disease prevention [121]. To our knowledge, no further studies have investigated why immune privilege in the ovary depends on the neonatal thymus but immune privilege in the testes does not. This observation may point to important female–male differences in peripheral CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation.

The spontaneous *Scurfy* mutation in mice led to the discovery of the genetic basis for dominant immunological self-tolerance. The X-linked *Scurfy* mutation causes a lethal autoimmune syndrome in neonatal male mice similar to the autoimmune disease observed in females and males lacking the *Ctla4* or *Tgfb* genes. Genetic analyses linked the *Scurfy* phenotype to the *Foxp3* gene [136,137], so named for its protein product, a new member of the forkhead/winged-helix family of transcription factors [138]. Mutations in the evolutionarily conserved human ortholog, *FOXP3*, are responsible for IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), a lethal autoimmune disease syndrome of young boys [139,140].

The Foxp3 protein specifies much of the transcriptional signature of CD4<sup>+</sup>CD25<sup>+</sup> Treg cells, now designated CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells [141,142]. Alone, Foxp3 is not sufficient to endow CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells with their suppressive function. Rather, Foxp3 influences the transcription of an unexpectedly large number of CD4<sup>+</sup> T cell genes through its interactions with other transcription factors. The liganded VDR is a positive transcriptional regulator of the *Ikzf2* [15], *Ctla4* [13], and *IL2ra* [86] genes encoding the Helios, CTLA-4, and IL-2RA proteins, respectively, which are among the signature CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell genes needed for suppressive function. The tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells arise in the thymus and function in central tolerance [121,143], whereas the pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells are induced in the periphery and function in peripheral tolerance and tissue repair [144,145]. Together, these cells enforce dominant tolerance to self, commensal microbes, and fetal antigens. Indeed, research in the EAE model demonstrated the direct involvement of the pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells within the CNS in both the natural recovery from EAE and protection from EAE induction [146]. Importantly, the CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells from MS patients exhibit reduced suppressive function compared with those from healthy controls [147–149].

#### 4.3 1,25(OH)<sub>2</sub>D<sub>3</sub> supports protective pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation

The CD4<sup>+</sup> T cell lineage is remarkably heterogenic. Many differentiated CD4<sup>+</sup> T cell subsets are recognized by the extrinsic cues they sense, the nuclear transcription factors they display, their dominant transcriptional signatures, the immune proteins they produce, and the specific forms of immune defense in which they participate [145,150]. Phenotypic malleability is a signature feature of these diverse CD4<sup>+</sup> T cell types. Individual CD4<sup>+</sup> T cells have been observed to change their phenotype in response to extrinsic cues and intrinsic metabolic states. Moreover, the clonal progeny of individual CD4<sup>+</sup> T cells have been observed to differ phenotypically from their parental clone. This elegant system of phenotypic malleability allows tissue-resident CD4<sup>+</sup> T cells to respond to local microenvironmental cues in a manner that is appropriate to the time and place [151,152].

Phenotypic malleability has been envisioned mechanistically in terms of a bistable state switching model [153]. In this model, there exist two polarized, self-reinforcing CD4<sup>+</sup> T cell states, each with a characteristic gene regulatory network and the capacity to oppose the other state. Cell extrinsic cues such as hormones, pathogens, cell–cell interactions, and soluble mediators serve as potential drivers of transdifferentiation. A complex web of integrated cytosolic signal transduction pathways emanates from local microenvironmental cues and converges in the CD4<sup>+</sup> T cell's nucleus. In response, the constellation of nuclear factors (e.g., chromatin remodelers, transcription factors) either maintains the existing gene regulatory network or initiates reprogramming such that the cell transforms through intermediate states into the alternative CD4<sup>+</sup> T cell state.

A striking example of CD4<sup>+</sup> T cell phenotypic malleability is the interconversion between the CD4<sup>+</sup> Th17 cells that are pathogenic in MS, and the pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells that limit CD4<sup>+</sup> Th17 cell pathogenicity and are dysfunctional in MS [154]. Signaling of 1,25(OH)<sub>2</sub>D<sub>3</sub> to VDR-expressing CD4<sup>+</sup> T cells has a well-established role as an extrinsic signal driving the transformation of CD4<sup>+</sup> Th17 cells into pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in humans and mice [153,155]. The rapidly emerging field of regenerative immunology has begun to document the benefits of CD4<sup>+</sup> T cell phenotypic malleability beyond immune surveillance by demonstrating the regenerative functions of tissue-resident pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells [144,145]. In a mouse model of MS, the CNS-resident pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells promoted myelin regeneration

independently of their immunoregulatory capacity [156]. Recent reviews summarize a wealth of information on the precise extrinsic cues, intracellular signal transduction pathways, metabolic states, transcription factor networks, and chromatin accessibility states involved in CD4<sup>+</sup> T cell phenotypic malleability [121,150].

The actions of 1,25(OH)<sub>2</sub>D<sub>3</sub> as a driver of CD4<sup>+</sup> Th17 cell transdifferentiation into pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells have been analyzed in human T cell activation cultures. Briefly, 1,25(OH)<sub>2</sub>D<sub>3</sub> and IL-2 addition to T cell activation cultures increased the pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cell frequency and function, particularly for female T cells [157–160]. Similarly, 25(OH)D<sub>3</sub> addition also increased the pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cell frequency and function if myeloid lineage cells were present to produce 1,25(OH)<sub>2</sub>D<sub>3</sub> [161]. These results show that the 1,25(OH)<sub>2</sub>D<sub>3</sub> and IL-2 promoted the genesis of new pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells and/or the transformation of residual CD4<sup>+</sup> Th17 cells into pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells. Recent studies probed the question of clonal derivation. When mature pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells were first removed, 1,25(OH)<sub>2</sub>D<sub>3</sub> and IL-2 addition to the residual T cells increased the pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cell frequency indicating transdifferentiation rather than expansion of alternate clones [162].

Complementary results regarding 1,25(OH)<sub>2</sub>D<sub>3</sub> as a driver of CD4<sup>+</sup> Th17 cell transdifferentiation into pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells were obtained in rodent EAE studies. In this model, administering a single 1,25(OH)<sub>2</sub>D<sub>3</sub> dose [163] or three daily 1,25(OH)<sub>2</sub>D<sub>3</sub> doses [122,163], starting within a day of antigen priming, induced a high frequency of pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells and prevented EAE disease. Conditional *Vdr* gene targeting in CD4<sup>+</sup> T cells completely abrogated this effect, demonstrating that these cells were direct targets of 1,25(OH)<sub>2</sub>D<sub>3</sub> action [81]. More recent research identified the source of the 1,25(OH)<sub>2</sub>D<sub>3</sub> that is utilized by VDR-expressing CD4<sup>+</sup> T cells in vivo. Supplementary vitamin D<sub>3</sub> inhibited EAE disease induction only in female mice [13]. In these mice, conditional *Cyp27b1* gene targeting in myeloid lineage cells completely blocked the appearance of CNS-infiltrating pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells and decreased vitamin D<sub>3</sub>-mediated protection from EAE disease. Conditional *Cyp27b1* gene targeting also decreased the CTLA-4 protein in the pCD4<sup>+</sup>FoxP3<sup>+</sup> Treg cells. These and other results [164] show that *Cyp27b1*-expressing microglia produced 1,25(OH)<sub>2</sub>D<sub>3</sub> for paracrine signaling to CNS-infiltrating CD4<sup>+</sup> T cells, and further, the *Ctla4* gene, like the *Foxp3* and *Ilkzf2* genes, appeared to be a target of 1,25(OH)<sub>2</sub>D<sub>3</sub> and VDR transcriptional control.

#### 4.4 Androgens and estrogens support protective pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation

Androgens and estrogens also influence pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation. Androgen hormone metabolism, the X-linked *AR* gene, and the functions of the nuclear *AR* in immune suppression have been reviewed elsewhere [165]. In mice, administration of dihydrotestosterone (dihydro-TST) increased EAE disease resistance [166–168]. Importantly, unlike testosterone (TST), dihydro-TST cannot be metabolized to E2. Furthermore, a small pilot study in men with RRMS demonstrated that TST treatment slowed brain atrophy on magnetic resonance imaging (MRI) [169,170]. Recent research demonstrated that conditional *Ar* gene targeting specifically in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells increased the inflammatory response of male mice to allergen challenge in an asthma disease [126]. Further experiments confirmed that TST-*AR* signaling in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells stabilized *Foxp3* protein expression and increased CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell function.

Estrogenic hormones and their nuclear hormone receptors, ER $\alpha$  and ER $\beta$ , have complex immunoregulatory roles that include support for pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation [91]. Pioneering animal modeling experiments initially demonstrated a striking ability of E2 to increase EAE resistance [171,172]. The E2 inhibited encephalitogenic T cell differentiation and trafficking into the CNS and exerted neuroprotective effects on axons and the myelin sheath. These E2 protective effects correlated with upregulation of *Foxp3* gene expression and pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation [173]. Gene targeting experiments revealed that ER $\alpha$  signaling in CD4<sup>+</sup> T cells was necessary and sufficient for E2-mediated upregulation of *Foxp3* gene expression and pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation [127].

The influences of estrogenic hormones on CD4<sup>+</sup> T cell differentiation in humans have been reviewed [91]. A consistent observation is that estrus E2 levels induced proinflammatory CD4<sup>+</sup> Th1 cells producing IFN- $\gamma$  by promoting *Ifng* gene transcription. An intriguing recent observation is that the peripheral blood pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell frequency in nonpregnant women was only one-third of the frequency observed in men [174]. Moreover, the *FOXP3* transcript abundance per cell in nonpregnant women was only one-third of the abundance observed in men. These and other data reinforce the view that estrus E2 levels support proinflammatory CD4<sup>+</sup> Th1 cell differentiation and provide only limited support for pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation.

Recent research has resolved the conflicting results reported for pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell fluctuations during

human pregnancy [130]. A comprehensive analysis of Treg cell dynamics in the peripheral blood of pregnant women was performed wherein naive  $tCD4^+$  Treg cells were identified as  $Foxp3^{lo}CD45RA^+$  and functionally suppressive  $pCD4^+Foxp3^+$  Treg cells were identified as  $Foxp3^{hi}CD45RA^-$  [133]. The intriguing results demonstrated that functionally suppressive  $pCD4^+Foxp3^+$  Treg cells decreased in peripheral blood to a nadir in the second and third trimesters, corresponding with the appearance of these cells at the maternal–fetal interface [131]. Simultaneously, thymic output of naive  $tCD4^+$  Treg cells, coupled with their increased longevity, replenished the peripheral naive  $tCD4^+$  Treg cell pool throughout pregnancy. In conclusion, pregnancy hormones support thymic naive  $tCD4^+$  Treg cell output and longevity and selective migration of mature  $pCD4^+Foxp3^+$  Treg cells to the maternal–fetal interface to enforce fetal tolerance.

Clearly, *FOXP3* and rodent *Foxp3* gene expression is crucial for the transdifferentiation of encephalitogenic  $CD4^+$  Th17 cells into dominant immunosuppressive  $pCD4^+Foxp3^+$  Treg cells. These cells promote myelin repair and regeneration in the CNS independently of their dominant immunosuppressive capacity. In the CNS tissue, the activated microglial cells are the source of the  $1,25(OH)_2D_3$  for paracrine signaling to CNS-infiltrating  $CD4^+$  T cells. Moreover, the  $pCD4^+Foxp3^+$  Treg cells are essential for successful female reproduction. The placenta is the source of the  $1,25(OH)_2D_3$  in pregnant females [175]. The  $1,25(OH)_2D_3$ , TST, and pregnancy-level E2 are independently capable of enhancing *FOXP3* and *Foxp3* gene transcription. The question arises, could differential  $1,25(OH)_2D_3$ , TST, and E2 enhancement of *FOXP3* gene transcription in males and females be a mechanism linking declining  $25(OH)D$  status with increasing MS risk in a female-biased manner?

## 4.5 Hormonal regulation of the *FOXP3* gene

The evidence presented before demonstrated that between the 1930 and 1989 birth cohorts, the overall MS incidence ratio increased, and further, the composite F:M MS incidence ratio increased from 1.9 to 4.6 in high latitude countries ( $>45$  degrees), and from 1.5 to 2.3 in low latitude countries ( $<45$  degrees) [94]. To achieve these ominous benchmarks, the underlying mechanisms would need to exhibit strong amplifying effects and functional impacts in multiple tissues. Collectively, the sexual dimorphism observations summarized in Table 101.1 implicate sex hormone involvement in one or more underlying mechanisms. Finally, the coincidental precipitous decline in population vitamin D status suggests the hypothesis that this may be a contributing factor [7].

There is abundant evidence for  $1,25(OH)_2D_3$ -mediated regulation of many immune system cells, CNS-resident cells, chemokines, cytokines, growth factors, cell surface receptors, costimulatory molecules, immune checkpoint molecules, signal transduction pathways, nuclear transcription factors, and epigenetic mechanisms. Given the myriad possibilities, we sought to develop a theoretical mechanism that might apply, and even if wrong, might serve as a prototypical mechanism to illustrate how reduced  $1,25(OH)_2D_3$  signaling in combination with sex hormone involvement could contribute to the growing F:M MS incidence ratio. To this end, we focused on regulation of the *FOXP3* gene.

The *FOXP3* protein directly transactivates hundreds of  $CD4^+$  T cell genes, some of which are themselves transcriptional activators [142]. Moreover,  $pCD4^+Foxp3^+$  Treg cells impact dozens of tissues [145]. Given these facts, it seems possible that differential  $1,25(OH)_2D_3$ -mediated regulation of the *FOXP3* gene in females and males might have a strong enough amplifier effect and a broad enough functional outcome to achieve the female MS gender bias that has been recorded. Consequently, we developed a hypothetical model for differential  $1,25(OH)_2D_3$ -mediated enhancement of *FOXP3* gene transcription in women and men. This model may be incorrect. Even so, it may be instructive in future searches for correct mechanisms.

Substantial evidence indicates that  $1,25(OH)_2D_3$  promotes encephalitogenic  $CD4^+$  Th17 cell transdifferentiation into immunosuppressive  $pCD4^+Foxp3^+$  Treg cells in part by enhancing *FOXP3* gene expression. *FOXP3* gene regulation involves epigenetic remodeling and topological association of activated enhancer domains with the gene promoter to set a transcriptional threshold. Several *FOXP3* enhancer domains harbor steroid hormone receptor-responsive elements that serve as de facto hormonal control regions for  $1,25(OH)_2D_3$ , TST, and E2. Here, we consider how the hormonal control regions might affect the topological association of enhancer domains with the promoter such that reduced  $1,25(OH)_2D_3$  signaling would have a more negative impact on *FOXP3* gene transcription and  $pCD4^+Foxp3^+$  Treg cells in females compared with males.

To develop the conceptual framework, we first review *FOXP3* gene structure and the *cis*-regulatory elements that activate the promoter [176]. We then summarize the interactions of the three nuclear steroid receptors, VDR [123], AR [125], and  $ER\alpha$  [130], with their respective hormonal control regions. Finally, we present a holistic model wherein  $1,25(OH)_2D_3$ –VDR signaling could be redundant in males due to the proximity of the VDR and AR response elements within a particular enhancer region that is crucial for  $CD4^+$  Th17 cell transdifferentiation and  $pCD4^+Foxp3^+$  Treg cell stability. Because this particular enhancer lacks an  $ER\alpha$  response element, the



1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling would be indispensable for its activation in females.

The highly conserved human *FOXP3* and murine *Foxp3* genes reside within syntenic X-chromosome regions. The promoter upstream of the first *FOXP3* coding exon controls gene expression in response to TCR and IL-2 signaling [177]. Comparative DNA sequence analysis of *Foxp3* genes in placental mammals identified four conserved, noncoding sequences, CNS0, CNS1, CNS2, and CNS3 (Fig. 101.1) [178–180]. The CNS0, CNS1, CNS2, and CNS3 regions encompass distal enhancers, also known as *cis*-regulatory elements [181].

The pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the periphery are regulatory chameleons [145]. They utilize signals present in their local tissue environment to adapt their transcriptional signature to perform tissue homeostatic functions that extend beyond immune tolerance. A cell's transcriptional identity is established and maintained by the specific activity of the *cis*-regulatory DNA elements, including promoters, where RNA polymerase II transcription initiation complexes assemble at the transcriptional start site (TSS), and enhancers that determine RNA polymerase II loading and/or release [182]. Signals present in the pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell's microenvironment determine which proteins are bound to the *Foxp3* gene's *cis*-regulatory elements. Enhancer–promoter connectivity is achieved by long-distance chromosomal DNA looping as protein-activated *cis*-regulatory elements and promoters come into close proximity with the TSS to form large topologically associated structures. An elegant analysis of chromosome conformation, chromatin accessibility, and Foxp3 binding to enhancer–promoter loops at the single cell level in mice with and without a functional *Foxp3* gene revealed that the Foxp3 protein directly transactivated hundreds of Treg-specific genes, including genes like *Irf2* that are themselves master transcriptional regulators [142]. The remarkable ability of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells to perform tissue homeostatic functions in addition to immune regulation reflects this complex transcriptional rewiring in response to signals present in the tissue microenvironment. Depending on the tissue, some of these signals will be hormonal in nature.

The *Foxp3* gene promoter is first activated in early stages of thymic development (Fig. 101.1A) [180,183,184]. The CNS0 region upstream of the *Foxp3* gene promoter and the intergenic CNS3 region downstream from exon 1 were necessary for this step, since their deletion resulted in a lethal autoimmune syndrome akin to the *Scurfy* phenotype. The CNS0 region did not demonstrate enhancer activity. However, prior to TCR signaling, it carried the H3K4me3 and H3K9/14Ac epigenetic marks indicative of a poised enhancer, suggesting it functions as an epigenetic switch to open the *Foxp3* locus for transcription. The pioneer factor SATB1

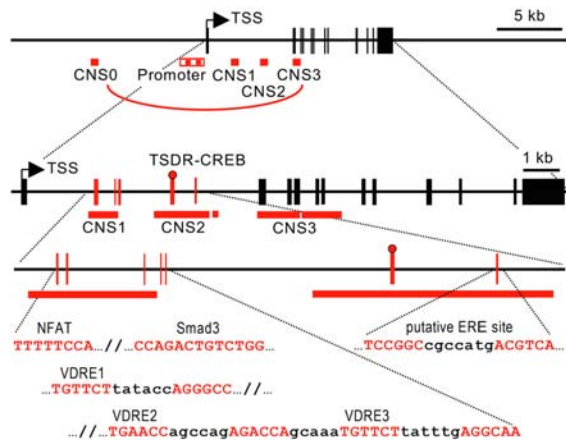
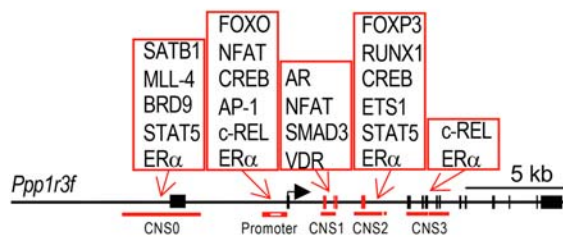
bound to a site in CNS0 (Fig. 101.1B). Disruption of the *Satb1* gene in T cells severely impaired *Foxp3* gene expression and CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell development. Subsequent TCR and CD28 signaling and c-REL binding to CNS3 initiated sequential DNA looping to bring the CNS0 and CNS3 regions together with the promoter in a topologically associated structure that culminated in *Foxp3* gene transcription.

The CNS1 enhancer region downstream of the promoter is important for extrathymic *FOXP3* gene expression, particularly in T cells associated with mucosal surfaces (Fig. 101.1B) [176,180,185]. CNS1 is not needed for tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell development. Transforming growth factor-beta (TGF-β) signals activate SMAD3 binding to a conserved site in CNS1 (Fig. 101.1C). Deleting the SMAD3 site decreased pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the gut-associated lymphoid tissue, but had no impact on tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell or pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation [186]. A recent review summarizes the copious literature on TGF-β-mediated control of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell transdifferentiation [187].

The CNS1 enhancer region is a very recent and remarkable addition to the genome of placental mammals [188]. It is completely missing in nonplacental mammals. An annotated SINE retrotransposon of the mammalian-wide interspersed repeat family encompasses most of the CNS1 enhancer region, suggesting a retrotransposon insertion event abruptly added the CNS1 enhancer to the *FOXP3* gene early in mammalian evolution. Its subsequent retention throughout eutherian mammalian evolution prompts the question does CNS1 contribute to the reproductive success of placental mammals? CNS1 deletion studies in mice suggested the answer was yes. Abundant offspring were produced when wild-type females mated with allogeneic or syngeneic males, and when CNS1-deficient females mated with syngeneic males. However, there was a high rate of embryo resorption when CNS1-deficient females mated with allogeneic males. Embryo resorption correlated with a paucity of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, and T cell infiltration and inflammatory pathology of the placenta. These findings indicate an aggressive T cell response to paternal antigens on the fetus. Thus, the CNS1 enhancer increased reproductive success by promoting pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell generation to mitigate maternal–fetal immune conflict.

Knowledge of the *FOXP3* gene CNS1 enhancer's contribution to female reproductive success suggests that hormones associated with the maternal–fetal interface may serve as cell-extrinsic enhancer activation signals. The 1,25(OH)<sub>2</sub>D<sub>3</sub> is one of these hormones [175]. Three vitamin D–responsive elements (VDREs) have been identified in the CNS1 region (Fig. 101.1D) [123]. The 1,25(OH)<sub>2</sub>D<sub>3</sub> addition to human primary CD25<sup>+</sup>CD4<sup>+</sup> T cell stimulation cultures yielded a dose-



A) The human *FOXP3* geneB) Factors that regulate *FOXP3* expression

## C) NFAT-Smad3 region sequence conservation in CNS1

Human 6984 ATTTTTCATTACTATAGAGGTTAAGAGTGGGTACTGGAGCAGACTGTCTGGGAC 7043  
 Mouse 7448050 ATTTTTCATTACTATAGAGGTTAAGAGTGGGTACTGGAGCAGACTGTCTGGGAC 7448109

## D) VDRE1 region sequence conservation in CNS1

Human 7500 AGTATCTGTTTATACAGGGCCACACTGTTTGTGATTGTTGTTTGCATACAAAT 7559  
 Mouse 7448547 AGTATCTGTTTATACAGGGCCACACTGTTTGTGATTGTTGTTTGCATACAAAT 7448605  
 Human 7560 CAATACCCAGCCATGGGTGCTCCCTGGCACCTTC 7593  
 Mouse 7448606 CAATA-CGAGCCATGGGTGCTCTGGCACCTAGC 7448638

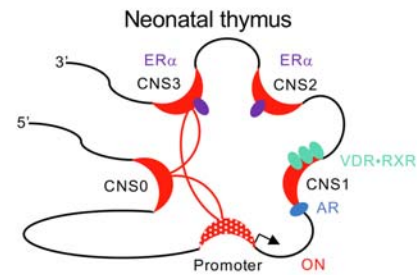
## E) ARE region upstream of CNS1

Human 6670 CATGTCTTACTCCTAATAGGGTGTTCATCTTATTTTCCC 6710

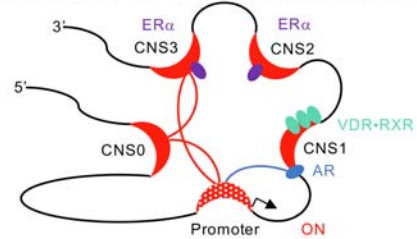
## F) ERE region sequence conservation in CNS2

Human 9019 GCATCCGGCCCATGACGTCAATGGCGGAAAAATCTGGGCAAGTCGGGGCTGTGACAA 9078  
 Mouse 7450370 GCATCCGGCCCATGACGTCAATGGCGGAAAAATCTGGGCAAGTCGGGGCTGTGACAA 7450428  
 Human 9079 CAGGGCCAGATGACAGCCCGATATGAAAAATAATCTGTGTCAGAGAAATCCCCCA 9138  
 Mouse 7450429 CAGGGCCAGATGACAGCCCGATATGAAAAATAATCTGTGTCAGAGAAATCCCCCA 7450488  
 Human 9139 TTCAGCTTCTGAGAAACCCAGTCAGAAAGGGAGCTCCCAACAGACAG 9185  
 Mouse 7450489 TTCAGCTTCTGAGAAACCCAGTCAGAAAGGGAGCTCCCAACAGACAG 7450533

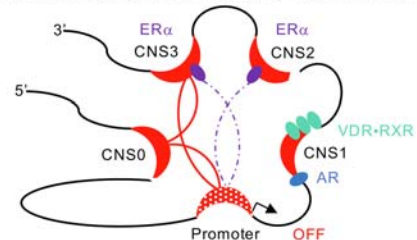
## G) Hormone-enhancer-promoter interactions



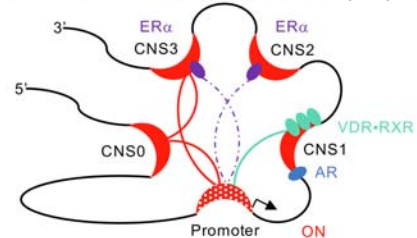
Adult male; TST 20 nmol/L, vitamin D deficient



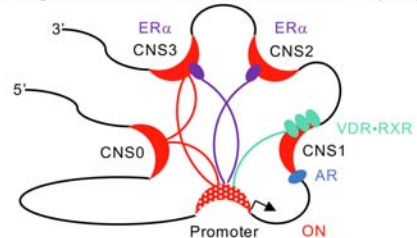
Adult female; E2 0.4 nmol/L, vitamin D deficient



Adult female; E2 0.4 nmol/L, 1,25-(OH)<sub>2</sub>D<sub>3</sub> 112 pmol/L



Pregnant female; E2 75 nmol/L, 1,25-(OH)<sub>2</sub>D<sub>3</sub> 260 pmol/L



**FIGURE 101.1** Human *FOXP3* gene transcriptional regulation and a hypothetical model to explain sexual dimorphisms in MS. (A) Genomic organization of the human *FOXP3* gene (NC\_000023.11). The transcriptional start site is labeled TSS. The eleven coding exons are shown as filled black boxes. The four conserved noncoding sequences are shown as filled red boxes numbered CNS0, CNS1, CNS2, and CNS3. The promoter region is shown as a checked red box. The red arc indicates a superenhancer formed by CNS0 and CNS3. TSDR, Treg-specific demethylated region; CREB, cyclic-AMP response element; NFAT, nuclear factor of activated T cells; VDRE, vitamin D response element; ERE,

dependent induction of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. Reporter assays comparing *FOXP3* promoter vectors with and without CNS1 demonstrated a threefold increase in promoter activity when 1,25(OH)<sub>2</sub>D<sub>3</sub> was added to these cultures. No VDRE activity was detected outside the CNS1 region. Further experiments identified VDR–RXR binding to DNA fragments from the CNS1 region. Systematic mutational analyses, VDR–RXR binding experiments, and promoter activation studies ultimately defined the three VDREs. The first of these, VDRE1, is 75% conserved between human and mouse (C.E.H. unpublished).

The three VDREs in the human *FOXP3* gene CNS1 region are important for successful pregnancy in women. The mother's serum 25(OH)D concentration correlated directly with the *FOXP3* transcript abundance and the pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell frequency in the placental tissue [189]. The placenta produces 1,25(OH)<sub>2</sub>D<sub>3</sub> [175]. This production was so robust that the mother's serum 1,25(OH)<sub>2</sub>D<sub>3</sub> level increased about 2.5-fold [117]. A recent review and metaanalysis calculated that miscarriage rates were nearly twofold higher in vitamin D–deficient women compared with vitamin D–replete women [190]. Together, the evidence suggests that vitamin D–replete status, placental 1,25(OH)<sub>2</sub>D<sub>3</sub> production, and VDR binding to the three VDREs in the human *FOXP3* gene's CNS1 enhancer are important for successful pregnancy. We refer interested readers to Chapter 32, "Vitamin D and Pregnancy," Chapter 34, "Vitamin D and the Placenta," and Chapter 39, "Vitamin D: Role in Reproductive Biology and Dysfunction in Women" for additional information on vitamin D and female reproduction.

Dihydro-TST also activates the human *FOXP3* gene CNS1 enhancer [125]. Reporter assays were performed in embryonic kidney cells (HEK-293) that were cotransfected with a *FOXP3* promoter vector with or without CNS1, and an AR expression construct. In this system, dihydro-TST addition increased promoter activity threefold only when the CNS1 region was present. Further experiments demonstrated AR binding to a DNA

fragment from the CNS1 region (Fig. 101.1E). Subsequent mutational analyses identified the 15 bp AR response element (ARE). Comparative DNA sequence analysis did not reveal conservation of this ARE in the mouse, but possible ARE half sites were observed (C.E.H. unpublished). Dihydro-TST also stimulated AR recruitment to the ARE in CNS1 of the *FOXP3* gene in primary human pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells from the peripheral blood of young men and women. The liganded AR binding increased acetylated-H4 in the area of the ARE, consistent with gene activation. This binding had no impact on two repressive epigenetic marks, methylation of H3K27 or methylation of DNA. Interestingly, *FOXP3* gene expression was more responsive to in vitro dihydro-TST addition in pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells from young women than young men. In the women, responsiveness of the *FOXP3* gene to dihydro-TST addition correlated with serum E2 and menstrual cycle stage. One interpretation of this gender-specific effect is that dihydro-TST activated the CNS1 enhancer that was dormant in pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells from young women but already activated in the cells from young men.

The CNS2 region in the first intron of the *FOXP3* gene exhibits strong enhancer activity. This region is noteworthy for a Treg-specific demethylated region (TSDR) observed in tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell development (Fig. 101.1A) [191,192]. The TSDR served as an epigenetic mark for stable tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, since these CpG sequences were fully methylated in effector CD4<sup>+</sup> Th17 cells, even those that transiently expressed *FOXP3* [193]. Deletional analysis revealed that CNS2 was not essential for *Foxp3* expression in the thymus, but was essential for stable *Foxp3* expression in mature, dividing tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells [180]. The CNS2 region is the site of an autoregulatory loop. The Foxp3 protein transactivated this CNS2 region in a Runx1-dependent manner to ensure stable *Foxp3* expression.

The CNS2 region is also noteworthy as an E2-responsive region. Potential E $\alpha$ -binding sites were mapped within the cis-regulatory elements of the

estrogen response element; ARE, androgen response element. (B) Factors that regulate *Foxp3* gene transcription are shown in red boxes with arrows pointing to the regions where the transcription factors bind. (C) The NFAT and SMAD3 target sequences within CNS1 are shaded in a sequence comparison between human and mouse. This region is 88% conserved. (D) The VDRE1 target sequence within CNS1 is shaded in a sequence comparison between human and mouse. This region is 85% conserved. (E) The ARE target sequence within CNS1 is shaded (NG\_007392.1; 6683–6697). The full ARE is not conserved in mouse although half elements may be present. (F) The ERE full target site and one ERE half site within CNS2 are shaded. The region encompassing the ERE full and half target sites is 87% conserved. The remaining six ERE half sites are not shown. (G) Hypothetical models to describe how hormonal control regions might affect the topological association of the CNS1 enhancer domain with the *FOXP3* gene promoter in T lymphocytes. From top to bottom, the illustrations show T lymphocytes in the neonatal thymus, or T lymphocytes in the periphery of the vitamin D–deficient adult male, the vitamin D–deficient adult female, the vitamin D–replete adult female, and the pregnant female. The black line represents the human *FOXP3* gene DNA. The red crescents represent the conserved noncoding sequences (solid red) and the promoter (red checkered). The solid red lines represent a topological association between the CNS0 and CNS3 regions and the gene promoter. The hormone control elements are depicted as colored ovals. These elements are labeled with their respective nuclear steroid receptors in the same color. The colored lines connecting the hormone control elements with the promoter represent a topological association. In the case of the ER $\alpha$ , the line is dot-dash to represent a weak interaction and solid to represent a strong interaction.

*FOXP3* gene using the ChIP-qPCR protocol applied to pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells isolated from the peripheral blood of healthy male donors [129]. The ER $\alpha$  binding was observed within the promoter, and in CNS2 and CNS3, but not CNS1. An in silico search for E2 response elements (EREs) suggested the existence of a full palindromic repeat and an ERE half site in CNS2, a half site in the promoter, two half sites downstream of the TSS, and two half sites in the CNS3 region (Fig. 101.1F). These putative EREs showed 100% sequence conservation between human and mouse. To our knowledge, no further experimentation has established the existence of these putative EREs, the E2 concentrations needed to promote ER $\alpha$  monomer binding, or the contribution of putative EAE half sites and ER $\alpha$  dimerization to *FOXP3* gene transcription.

Assuming the temporal and geographic correlation between decreasing 25(OH)D levels and increasing female MS prevalence represents a causal relationship, we sought a biochemical model to explain this and other MS sexual dimorphisms. The search for a model focused on the CD4<sup>+</sup> Th17 cell to pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell transformation, the *FOXP3* gene, and the *cis*-regulatory elements that interact with the gene promoter to set the transcriptional threshold. Before, we reviewed preliminary reports that 1,25(OH)<sub>2</sub>D<sub>3</sub>, TST, and E2 exert hormonal control over *FOXP3* gene transcription. The placement of the hormonal control regions within the *cis*-regulatory elements of the human *FOXP3* gene is significant. Three VDREs and an ARE, but no EREs, map within the CNS1 region, whereas a putative ERE and an ERE half site, but no VDREs or AREs map within the CNS2 region (Fig. 101.1B–F). The model considers how these hormonal control regions might affect the topological association of the CNS1 enhancer domain with the promoter such that reduced 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling would have a greater negative impact on *FOXP3* gene transcription and pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell stability in females than males.

The model is presented in Fig. 101.1G. The promoter and the four conserved *cis*-regulatory elements are depicted as crescents arranged in a circle, with the DNA strand from 5' to 3' looping out between them. The VDR, the AR, and ER $\alpha$  proteins are labeled adjacent to colored ovals that represent their respective hormone-responsive elements. The uppermost drawing illustrates the core enhancer–promoter topological associations needed for *FOXP3* gene transcription initiation in the neonatal thymus. The red lines connecting the promoter with the CNS0 and CNS3 *cis*-regulatory elements indicate this topological association. For simplicity, the contributions of TCR-CREB, IL-2-STAT5, and TGF- $\beta$ -SMAD3 signaling to *FOXP3* promoter activation are assumed but not shown. Rather, colored lines are shown connecting similarly colored ovals to the promoter to

illustrate the contributions of 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR, TST-AR, and E2-ER $\alpha$  signaling to *FOXP3* promoter activation. The model presumes that *Cyp27b1*-expressing microglia produce 1,25(OH)<sub>2</sub>D<sub>3</sub> for highly localized paracrine signaling to CD4<sup>+</sup> T cells infiltrating the brain and spinal cord.

The vitamin D–deficient adult male illustration is below the neonatal thymus. The model proposes that TST-ARE signaling would activate the CNS1 enhancer region to join the topological association of enhancer domains with the promoter. This CNS1 enhancer activation would occur independently of 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling in males. Thus, the model predicts that because their respective hormone-responsive elements are proximal, the TST-AR and 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signals would be redundant for promoting CNS1 enhancer–promoter interactions. Thus, the TST and 1,25(OH)<sub>2</sub>D<sub>3</sub> are predicted to be redundant for driving the CD4<sup>+</sup> Th17 cell to pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell transformation in the male brain and spinal cord.

The vitamin D–deficient adult female illustration is below the vitamin D–deficient adult male illustration. The model proposes that in vitamin D–deficient females, E2-ER $\alpha$  signaling cannot activate the CNS1 enhancer region to join the enhancer–promoter cluster because there is no putative ERE in CNS1. Although there are putative ERE and ERE half sites in CNS2 and CNS3, estrus E2 levels are too low to activate these *cis*-regulatory elements further, and these elements do not contribute to *FOXP3* transactivation. Moreover, due to vitamin D deficiency, microglia would not have enough 25(OH)D substrate to produce 1,25(OH)<sub>2</sub>D<sub>3</sub> for VDR signaling to activate the CNS1 enhancer region. Thus, the model predicts that in vitamin D–deficient females, the CD4<sup>+</sup> Th17 cells infiltrating the brain and spinal cord for any reason would remain unchecked to orchestrate autoimmune-mediated tissue damage.

The vitamin D–replete adult female illustration is below the vitamin D–deficient female illustration. The model proposes that in these replete females, the *Cyp27b1*-expressing microglia would produce enough 1,25(OH)<sub>2</sub>D<sub>3</sub> for VDR signaling to activate the CNS1 enhancer region to join the topological association of enhancer domains with the promoter. Thus, the CD4<sup>+</sup> Th17 cells infiltrating the brain and spinal cord for any reason would be driven to transdifferentiate into pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells that protect the tissue from autoimmune-mediated damage and promote tissue repair and regeneration.

Finally, an illustration of *cis*-regulatory element interactions with the *FOXP3* promoter in pregnant females is at the bottom of Fig. 101.1G. The model suggests that pregnancy E2 levels would strengthen the CNS2 and CNS3 *cis*-regulatory element interactions with the promoter to maintain thymic output of tCD4<sup>+</sup>Foxp3<sup>+</sup> Treg

cells as observed [133]. Moreover, the model suggests that pregnancy 1,25(OH)<sub>2</sub>D<sub>3</sub> levels would strengthen the CNS1 interaction with the enhancer–promoter complex to promote the *FOXP3*-dependent transformation of CD4<sup>+</sup> Th17 cell into pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. This result would improve protection and myelin repair and regeneration in the brain and spinal cord as was observed [156].

This hypothetical model for differential 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated enhancement of *FOXP3* gene transcription in women and men has significant limitations. Foremost among them is the lack of evidence for a causal link between the three VDREs and the ARE in the CNS1 region and the transdifferentiation of encephalitogenic CD4<sup>+</sup> Th17 cells into protective pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in vivo. This is a particularly difficult question to address because VDRE1 shows only partial sequence conservation, and the ARE has no equivalent in the mouse. Without response element conservation, straightforward deletional analyses like those that established the role of the SMAD3 site for pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell stability in the gut-associated lymphoid tissue are not feasible. Unlike the VDRE and ARE sites, the full ERE and ERE half site in CNS2, and the ERE half sites in the promoter, near the TSS, and in the CNS3 region showed 100% sequence conservation between human and mouse. However, in silico analysis suggested the existence of these ERE and ERE half sites, but appropriate experimentation has not demonstrated their function in vitro or in vivo. In summary, there is as yet no in vivo evidence that the VDREs, the ARE, and the EREs have a direct role in *Foxp3* gene transactivation. In the absence of such evidence, it is not possible to evaluate experimentally whether the VDREs and the ARE are redundant in males, and whether the contribution

of the VDREs is essential in females as envisioned in the hypothetical model.

Despite these limitations, the hypothetical model has significant strengths. Foremost among the strengths is the conceptualization of a biochemical mechanism that would explain most if not all of the puzzling MS sexual dimorphisms that have been observed. The idea of a *cis*-regulatory element that can be activated by either 1,25(OH)<sub>2</sub>D<sub>3</sub> or TST in men but only by 1,25(OH)<sub>2</sub>D<sub>3</sub> in women for *FOXP3* expression is sound and conceptually useful in the effort to understand how the loss of 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling could elevate female MS disease risk 4.6-fold compared with men. This model is also consistent with all of the observed MS sexual dimorphisms listed in Table 101.1, and with the pregnancy data. In fact, the model predicts the unanticipated 4.6-fold increased MS disease risk in transsexual females compared with transsexual males. Indeed, the model predicts that elevating an individual's vitamin D status to support 1,25(OH)<sub>2</sub>D<sub>3</sub> signaling before administering hormonal treatments for gender incongruence might reduce the MS disease risk in transsexual females. Lastly, the model predicts that for an individual whose risk score for MS is high [194,195], an effort to evaluate and improve the individual's vitamin D status early on might be rewarded by a divergence between predicted MS risk and observed MS diagnosis rate (Box 101.1).

## 5. Interplay between vitamin D and CD4<sup>+</sup> type 1 regulatory T cells in MS

The vitamin D system has long been known for its immunoregulatory effects, and much of the focus to elucidate the mechanisms of 1,25(OH)<sub>2</sub>D<sub>3</sub> action has

### BOX 101.1

#### Recommendations from the vitamin D perspective concerning MS risk.

##### Which individuals warrant concern?

Individuals who experience neurological symptoms consistent with MS (e.g., episodic vision loss or double vision, sensory symptoms such as numbness or tingling, limb weakness, incoordination, impaired balance or gait, urinary symptoms).

Individuals who have a biological first-degree relative diagnosed with MS.

Individuals with a genetically predicted high risk of MS.

##### What steps can be considered?

Measure circulating 25(OH)D levels to establish a baseline; if this level is low according to the Endocrine Society Guidelines, consider increasing vitamin D<sub>3</sub> nutriture, then retest after a few months (see Chapter 57).

Consider offering educational information on early neurological signs consistent with MS and encourage discussions with the primary care physician if neurological disability signs arise.



been on the downregulation of CD4<sup>+</sup> T effector cell subsets and the increase in pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, as further discussed before. Another key subset involved in maintaining immune tolerance consists of CD4<sup>+</sup> type 1 regulatory T cells (Tr1), which are characterized by secretion of the immunosuppressive cytokine IL-10. In the previous version of this chapter, the role of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the regulation of IL-10 and Tr1 was discussed. The crucial role of IL-10 in protecting the CNS from inflammation had been well established using animal models of MS. In these models, IL-10 and IL-10R were necessary for protection. Furthermore, human data using T cells from MS patients showed that the CD4<sup>+</sup> Tr1 cells produced less IL-10 than healthy control T cells.

Notably, one pathway of interest from the vitamin D perspective was initiated by the discovery that CD46 ligation caused the IFN $\gamma$ -producing CD4<sup>+</sup> T helper 1 (Th1) cells to transdifferentiate from their proinflammatory phenotype to the IL-10-producing CD4<sup>+</sup> Tr1 cell phenotype. Human CD46 is a transmembrane protein expressed on all nucleated cells that is a central component of the innate immune system; it binds complement fragments C3b and C4b and aids in their inactivation [196]. It also functions as a CD4<sup>+</sup> T cell costimulatory molecule, as summarized in a recent review [197].

A pioneering discovery was the finding that MS patients exhibited a defect in CD46-induced CD4<sup>+</sup> Tr1 cells, as characterized by an impaired production of IL-10 [198–201]. This defect was also observed in a primate EAE model [202]. Moreover, two first reports had shown that dynamic processing of CD46 provided a molecular rheostat to control human T cell activation [203], and further, that addition of 1,25(OH)<sub>2</sub>D<sub>3</sub> partially restored the dysfunctional transdifferentiation switch in MS patient CD4<sup>+</sup> Th1 cells, at least in vitro [160]. Whether this was occurring in vivo was unknown. In this updated chapter, we first summarize the previous data on IL-10, CD4<sup>+</sup> Tr1 cells, and MS and how 1,25(OH)<sub>2</sub>D<sub>3</sub> contributes to CD4<sup>+</sup> Tr1 cell functions. We then discuss novel data suggesting that indeed, 1,25(OH)<sub>2</sub>D<sub>3</sub> acts on the CD46 pathway in CD4<sup>+</sup> T cells from patients with chronic inflammation.

## 5.1 IL-10, CD4<sup>+</sup> Tr1 cell discovery and characteristics

In 1997, a CD4<sup>+</sup> T cell population secreting IL-10, a potent antiinflammatory cytokine, was first reported [204]. These cells secreted mainly IL-10 and some TGF $\beta$ , but lower levels of the other cytokines including IFN $\gamma$ . The T cells secreting this particular cytokine profile could be generated in both mice and humans. Named CD4<sup>+</sup> Tr1 cells, they were proposed to

participate in immune tolerance through the secretion of IL-10 [205]. Of note, although it is widely accepted that IL-10 is mainly an antiinflammatory cytokine, recent studies highlight the proinflammatory role IL-10 may also play when produced by effector T cells, especially CD4<sup>+</sup> Th1 cells, because it promotes their survival in the CNS [206]. It is likely that the source of IL-10, the timing, and the local microenvironment impact its function. This discovery opened up a novel area of investigation to fully understand the role of this potent cytokine. Interested readers are encouraged to see a recent review for more details [207]. Nevertheless, the predominant role of CD4<sup>+</sup> Tr1 cells is to reduce tissue inflammation and autoimmunity by suppressing effector T cell functions [208]. A lack of suitable identifying markers has long hampered the study of CD4<sup>+</sup> Tr1 cells [209,210]. Transcriptomics analysis of IL-10-secreting T cells showed their heterogeneity. However, current methods define CD4<sup>+</sup> Tr1 cells with the highly suppressive phenotype by expression of several immune checkpoint inhibitors (LAG-3, PD-1, CTLA-4, TIM-3, TIGIT), the chemokine receptor CCR5, the integrin CD49b, and LAG3 on IL-10-producing Foxp3<sup>+</sup> CD4<sup>+</sup> T cells [211].

## 5.2 CD4<sup>+</sup> Tr1 cells and MS

As discussed in the previous version of this chapter, several studies have highlighted the key role of the IL-10-producing regulatory T cells in the murine EAE model of MS. The essential role of IL-10 has clearly been demonstrated by the initial studies showing that IL-10-deficient mice exhibit significantly increased EAE severity [212]. In contrast, mice expressing the human IL-10 transgene under the control of a class II major histocompatibility complex (MHC) promoter are resistant to EAE [213]. Hence the IL-10/IL-10R axis is necessary to protect the CNS from inflammation in this model.

In MS, increased IL-10 production is associated with remissions and is induced by IFN $\beta$  treatment [208]. Importantly, myelin-reactive T cells from healthy donors produced more IL-10 than MS patient T cells [214], supporting the view that IL-10 suppressed autoreactive T cells in healthy donors and was indeed critical in the maintenance of immune homeostasis. A novel study following the percentage of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and CD4<sup>+</sup> Tr1 cells in MS patients receiving IFN $\beta$  treatment reported that the CD4<sup>+</sup> Tr1 cell frequency constantly increased in patients with stable MRI scans, while patients with new lesions exhibited an increase in the percentage of Tr1 only in the first year of therapy [200].

Hence, manipulating CD4<sup>+</sup> Tr1 cells as therapeutic agents to restore tolerance is actively being investigated

[208]. The cytokine IL-27 induced CD4<sup>+</sup> Tr1 cells in both mice and humans [215–217]. In humans, CD4<sup>+</sup> Tr1 cells were also induced by coligation of the TCR and CD46, which induced CD4<sup>+</sup> Th1 cell differentiation, followed by a switch from the proinflammatory Th1 phenotype to the CD4<sup>+</sup> Tr1 phenotype, characterized by increased IL-10 production and reduced IFN $\gamma$  secretion [218,219]. This approach is ineffective in mice, because CD46 expression is restricted to cells in the testis and the eyes. In addition to its complement receptor function, CD46 acts as a docking receptor for many pathogens [220], including two viruses associated with MS, human herpes-virus 6 (HHV-6) [221,222], and cytomegalovirus (CMV) [223]. Ligation of CD46 on CD4<sup>+</sup> Tr1 cells serves as a link between innate and adaptive immunity, since it controls T cell activation, differentiation, polarity, and metabolism, as further discussed in recent reviews [197,224].

Two crucial discoveries focused intense interest on CD4<sup>+</sup> Tr1 cells in the context of vitamin D and MS. Firstly, MS patients exhibited a defect in CD46-induced Tr1 cells that were characterized by impaired production of IL-10 upon CD46 activation [198–201]. Secondly, 1,25(OH) $_2$ D $_3$  treatment of CD4<sup>+</sup> T cells from MS patients and healthy controls profoundly amplified the IL-10-producing phenotype of CD46-costimulated CD4<sup>+</sup> T cells, while concomitantly decreasing IFN $\gamma$  production [160]. This transdifferentiation was further described as a 1,25(OH) $_2$ D $_3$ -driven biological switch (see Fig. 101.2 in Ref. [153]). The bistable switch model envisioned the CD4<sup>+</sup> Th1 cells and CD4<sup>+</sup> Tr1 cells as two immune states, proinflammatory and antiinflammatory, each of which is characterized by a particular gene regulatory network that enables the cell to respond to external stimuli in a biologically appropriate manner. Each type of cell produces an autocrine growth factor that reinforces its own survival. Moreover, each type of cell produces a cytokine that impedes transdifferentiation to the opposing cell type. External microenvironmental stimuli, for example, 1,25(OH) $_2$ D $_3$ , other hormones, pathogen-associated molecular patterns, cytokines, metabolites, and cell–cell interactions, serve as switch inputs to drive transdifferentiation to the alternate T cell state. The biological benefit of such a switch is that it permits a rapid reprogramming to an alternative gene regulatory network, possibly by epigenetic mechanisms, allowing a quick and appropriate response [153].

Mechanistically, it was shown that TCR activation led to a change in the glycosylation of CD46 that favored the recruitment of CD46 to the immune synapse, a key step for its regulatory function, and these glycosylation changes appear to be dysregulated in MS T cells [203]. Importantly, previous research demonstrated a gene–environment interaction mechanism in MS that involved dysregulated N-glycosylation of membrane

proteins [225]. In MS, interleukin-7 receptor variants led to MGAT1 downregulation and a failure of membrane protein N-glycosylation. Testing this mechanism by gene targeting in the EAE model revealed an essential role for the N-glycan branching enzyme, MGAT1, to prevent T cell hyperactivity and spontaneous inflammatory demyelination and neurodegeneration. Testing this mechanism in vitro with activated T cells from MS patients demonstrated that 1,25(OH) $_2$ D $_3$  addition significantly enhanced *MGAT1* transcripts, counteracted MGAT1 downregulation, and restored membrane protein N-glycosylation. The involvement of 1,25(OH) $_2$ D $_3$  in the glycosylation of CD46 that enhances its recruitment to the immune synapse clearly warrants further study in the context of improving CD4<sup>+</sup> Tr1 cell function in MS patients.

Increased levels of soluble CD46 are found in the serum and CSF of MS patients, which may correlate with the activation state of their T cells [226]. It is possible that HHV-6 triggers the CD46 pathway in MS, since associations between HHV-6 infection and active MS have been described, and complexes of HHV-6 bound to soluble CD46 have been isolated from MS sera [227,228]. Many if not most MS patients have low vitamin D status including among others those of European [229,230], African [231], Hispanic [232], and Iranian [233] descent. Together, these observations suggest the hypothesis that virus-mediated triggering of the CD46 pathway in individuals with low vitamin D status might lead to robust CD4<sup>+</sup> Th1 cell differentiation and proinflammatory function that is not properly regulated by CD4<sup>+</sup> Tr1 cell differentiation and function.

### 5.3 1,25(OH) $_2$ D $_3$ modulation of IL-10 and CD4<sup>+</sup> Tr1 cell production

The actions of 1,25(OH) $_2$ D $_3$  with respect to human CD4<sup>+</sup> Tr1 cells have been studied intensively in vitro. The addition of 1,25(OH) $_2$ D $_3$  promoted the CD46 shedding that is required for its regulatory function [203,234]. It also enhanced CD4<sup>+</sup> Tr1 cell differentiation and suppressive function, suggesting that the hormone's antiinflammatory functions occur in part through its effects on CD46 [160]. Furthermore, in MS T cells, 1,25(OH) $_2$ D $_3$  partially restored the CD4<sup>+</sup> Th1 to CD4<sup>+</sup> Tr1 cell switch in vitro and the suppressive ability of these cells [160]. It was proposed that 1,25(OH) $_2$ D $_3$  could act on this bistable Th1/Tr1 switch by altering the dynamics of the system and driving the transition to the Tr1 biological state [153]. These data suggested the existence of cross-talk between the CD46 and 1,25(OH) $_2$ D $_3$  pathways. The 1,25(OH) $_2$ D $_3$  exerts its effects through the VDR, a nuclear hormone receptor

that heterodimerizes with RXR to regulate transcription of target genes (see Chapters 10 and 11).

Importantly, T cell receptor (TCR) signaling via the p38 pathway in naive human T cells induced VDR expression, which then transcriptionally activated the *PLCG1* gene encoding phospholipase C gamma 1 ~ 75-fold [235]. This phospholipase uses calcium as a cofactor to produce inositol 1,4,5-triphosphate, a transducer of receptor-mediated tyrosine kinase signals. The higher *PLCG1* gene expression amplified the effect of further TCR signaling for T cell activation. Novel data show that increased VDR expression is observed upon CD46 T cell costimulation, and more so than in T cells classically activated by CD28 [14]. Furthermore, CD46-costimulated T cells express the  $1\alpha$ -hydroxylase enzyme that produces  $1,25(\text{OH})_2\text{D}_3$  so they are able to utilize the hormone precursor,  $25(\text{OH})\text{D}$  [236]. The cross-talk between CD46 and VDR- $1,25(\text{OH})_2\text{D}_3$  signaling is further evidenced by the striking increase in 24-hydroxylase (which inactivates  $1,25(\text{OH})_2\text{D}_3$ ) by longer-term CD46 costimulation of T cells in the presence of  $1,25(\text{OH})_2\text{D}_3$  [14]. Increased 24-hydroxylase expression is crucial in an autocrine feedback loop that shortens the half-life of bioactive  $1,25(\text{OH})_2\text{D}_3$ , thereby limiting the duration of the hormone response (see Chapter 5). Furthermore, analysis of ex vivo T cells from MS patients receiving supplementary vitamin D exhibited similar phenotypes to what was observed in vitro, suggesting the in vivo activation of the CD46 pathway in humans [14].

These data were further corroborated in a recent study analyzing patients with severe COVID-19, including life-threatening hyperinflammation and acute respiratory distress syndrome (ARDS) [236]. These patients exhibited an enhanced  $\text{CD4}^+$  Th1 cell response with increased  $\text{IFN}\gamma$  but very low IL-10 levels, and enhanced complement response, notably of C3. Together, these observations suggested the  $\text{CD4}^+$  Th1 cells from the COVID-19 patients might be incapable of undergoing the CD46-mediated switch from the proinflammatory  $\text{CD4}^+$  Th1 cell phenotype to the anti-inflammatory  $\text{CD4}^+$  Tr1 cell phenotype for reasons that are not yet clear. In this study, CD46 costimulation of healthy T cells also resulted in increased VDR and  $1\alpha$ -hydroxylase enzyme expression, and this depended on cleavage of intracellular C3. Further experiments characterized the effects of  $1,25(\text{OH})_2\text{D}_3$  on transcriptomic and epigenetic changes in T cells activated by CD28 costimulation. Importantly, CD28 costimulation indirectly activates CD46 by cleavage of C3. Adding  $1,25(\text{OH})_2\text{D}_3$  to the CD28-costimulated T cells induced not only IL-10 but also IL-6, considered a proinflammatory cytokine. The  $1,25(\text{OH})_2\text{D}_3$  promoted IL-10 in an IL-6-STAT3 dependent manner, in agreement with a previous animal study showing that IL-6 promoted IL-10-

producing  $\text{CD4}^+$  T cells with Tr1-like characteristics [237].

To probe the mechanism of VDR action in the  $\text{CD4}^+$  T cells, the epigenetic changes were analyzed using H3K27Ac, a marker of active genome regions, in CUT&RUN experiments. They observed the modulation of superenhancers, allowing the binding of several transcription factors, such as c-JUN, STAT3, and BACH2, which together with VDR shaped the transcriptional response to VDR- $1,25(\text{OH})_2\text{D}_3$  signaling [236]. Importantly, the CD46-stimulated molecular pathways governing termination of proinflammatory  $\text{CD4}^+$  Th1 cell responses were impaired in the  $\text{CD4}^+$  T cells obtained from the bronchoalveolar lavage fluid of patients with severe COVID-19. It is likely that this impairment contributed to hyperinflammation and ARDS. Of note, in this study, no enrichment of  $\text{CD4}^+$  Tr1-specific genes was observed upon  $1,25(\text{OH})_2\text{D}_3$  addition, but this may be due to using CD28 stimulation rather than CD46-stimulation prior to the transcript analysis. Indeed, the analysis of T cells from MS patients who received supplementary vitamin D<sub>3</sub> or placebo clearly showed a preferential effect of  $1,25(\text{OH})_2\text{D}_3$  addition on CD46-activated T cells rather than CD28-activated T cells [14]. Further analysis of  $1,25(\text{OH})_2\text{D}_3$ -mediated effects directly on the CD46 pathway in the  $\text{CD4}^+$  Th1 cells from the bronchoalveolar lavage fluid of COVID-19 patients is urgently needed. Such analysis could yield novel mechanistic insights into how  $1,25(\text{OH})_2\text{D}_3$  directly supports switching of  $\text{CD4}^+$  Th1 cells into the  $\text{CD4}^+$  Tr1 cell phenotype. New insights might reveal why switching of pathogenic  $\text{CD4}^+$  Th1 cells into protective  $\text{CD4}^+$  Tr1 cells appears to be impaired and suggest new approaches to resolve hyperinflammation and ARDS in COVID-19 patients.

Overall, these two studies strongly emphasize the key role of the cross-talk between the CD46 pathway and VDR- $1,25(\text{OH})_2\text{D}_3$  signaling for control of hyperinflammation in humans. Recent work suggests a sufficient vitamin D status before an infection is more important than vitamin D<sub>3</sub> supplementation after infection [238]. Many clinical trials have begun to evaluate the effect of vitamin D<sub>3</sub> supplementation in patients with severe COVID-19. We are aware of the following trials registered at Clinicaltrials.gov: NCT04344041, NCT04411446, NCT04449718, NCT04525820. We refer interested readers to Chapter 99 for further details.

#### 5.4 $1,25(\text{OH})_2\text{D}_3$ as a potential $\text{CD4}^+$ Tr1 cell inducer in MS patients

Due to the highly suppressive function of  $\text{CD4}^+$  Tr1 cells, they are currently being investigated as a potential therapeutic in MS [208]. Since the biologically active



hormone  $1,25(\text{OH})_2\text{D}_3$  triggers  $\text{CD4}^+$  Tr1 cell differentiation in vitro, the question arises could biologically *inactive* vitamin  $\text{D}_3$  (cholecalciferol) supplementation be used to support this  $\text{CD4}^+$  Tr1 cell response in MS patients? In MS, vitamin  $\text{D}_3$  supplementation is safe and has been associated with a modulation of T cell responses. Data on vitamin  $\text{D}_3$  supplementation and immune system responses in vivo as well as effects on MS progression are, however, not consistent.

## 5.5 Summary of the interplay between $1,25(\text{OH})_2\text{D}_3$ and $\text{CD4}^+$ Tr1 cells in MS

It is clear that  $1,25(\text{OH})_2\text{D}_3$  exerts profound effects on cytokine production, promotes the regulatory  $\text{CD4}^+$  Tr1 cell subset, and controls the switch from production of  $\text{IFN}\gamma$  to IL-10. Recent autoantigens such as RASGRP2 have been identified in MS [239,240]. These autoantigens favor autoreactive Th1 cell migration to the brain. Switching these autoreactive  $\text{CD4}^+$  Th1 cells to an IL-10-producing phenotype may therefore dampen the local inflammation in the CNS. It was recently reported that  $1,25(\text{OH})_2\text{D}_3$  favored motility of  $\text{CD46}$ -costimulated T cells toward CXCL11, a ligand of CXCR3 expressed by Th1 cells and driving their migration to the CNS, suggesting enhanced migration of these Th1-switched Tr1 cells toward the CNS [14]. Locally increased IL-10 production might also be beneficial because it regulates microglial activation [164,241]. Therefore,  $1,25(\text{OH})_2\text{D}_3$ -mediated induction of  $\text{CD4}^+$  Tr1 cells in the CNS may also reduce local inflammation through this effect. The  $1,25(\text{OH})_2\text{D}_3$  has been shown to restore the altered  $\text{IFN}\gamma/\text{IL-10}$  switch in  $\text{CD4}^+$  T cells from patients with MS in vitro, and analysis of  $\text{CD4}^+$  T cells from patients supplemented with vitamin  $\text{D}_3$  suggests that the regulatory  $\text{CD46}$  pathway is activated in vivo by the vitamin D pathway.

In mice, an elegant paper showed expansion of  $\text{CD4}^+$  Tr1 cells using nanoparticles coated with autoimmune disease—relevant peptides bound to major histocompatibility complex class II molecules in different mouse models [242]. A combination with vitamin  $\text{D}_3$  may provide a further approach to switch on the IL-10 suppressive program in patients. Because analysis of the human brain is limiting, novel approaches such as the use of brain spheres or organoids may provide a suitable model to study the role of vitamin D compounds in modulating brain and immune cells, as well as their interaction, to provide a full picture of how it modulates brain inflammation.

Mechanistically, there are still many unknowns. Key roles for the transcription factors eomesodermin [243] and Bhlhe40 [244] in controlling the switch from  $\text{IFN}\gamma$  to IL-10 production have been reported. These reports

raise the question whether either of these transcription factors might be involved in the  $1,25(\text{OH})_2\text{D}_3$ -mediated IL-10 switch. Given the demonstrated safety of clinical trials involving vitamin  $\text{D}_3$  supplementation in MS patients, and the overall beneficial outcome in terms of  $1,25(\text{OH})_2\text{D}_3$ –VDR signaling and  $\text{CD4}^+$  T cell activation observed in vitro, it is likely that vitamin  $\text{D}_3$  will benefit MS patients with hypovitaminosis D, although timing early in the disease course may be a key to efficacy.

## 6. Oligodendrocytes, DNA methylation, and the methionine cycle

### 6.1 The vitamin D system supports oligodendrocyte myelin repair and replacement

Interest in vitamin D's neuroprotective effects on mature oligodendrocytes (OLGs) and myelin synthesis was surging when the previous version of this chapter was written. Pioneering experiments performed decades ago hinted at vitamin D's role in mammalian CNS development, function, and repair. The rate-limiting enzyme for  $1,25(\text{OH})_2\text{D}_3$  biosynthesis ( $1\alpha$ -hydroxylase) had been identified in brain sections [74,245], radiolabeled  $1,25(\text{OH})_2\text{D}_3$  binding to multiple sites in the mammalian brain had been demonstrated [246], and  $1,25(\text{OH})_2\text{D}_3$ -mediated stimulation of neurotrophin synthesis had been observed [247,248]. These early studies suggested that  $1,25(\text{OH})_2\text{D}_3$  could be considered a neurosteroid hormone. For more information on these topics, we refer interested readers to Chapter 27 (Vitamin D Brain Development and Function).

The colocalization of the VDR and myelin basic protein (MBP) staining in OLG of the brain and spinal cord prompted the hypothesis that  $1,25(\text{OH})_2\text{D}_3$ –VDR signaling might support myelin biosynthesis [249]. This hypothesis triggered seminal studies on demyelination and remyelination in the MS brain [250]. Serial MRI of RRMS patients revealed a seasonal periodicity in brain demyelinated lesion formation. New lesion numbers were 5.3-fold higher in April than October. Application of similar regression models simultaneously to demyelinating lesions and circulating  $25(\text{OH})\text{D}$  levels in the population showed that both variables varied by season [251]; the two curves showed an inverse correlation, with circulating  $25(\text{OH})\text{D}$  changes leading lesion number changes by  $\sim 2$  months. An MRI study performed a decade later produced an elegant polar plot where new RRMS brain T2 lesion formation was superimposed on ambient solar radiation data [252]. The plot revealed new lesion accrual increased significantly in the Spring after a winter of low solar radiation, and decreased abruptly in



September after a summer of high solar radiation. Published data on circulating 25(OH)D levels in this population [253] also showed an inverse correlation, with circulating 25(OH)D changes leading lesion number changes by  $\sim 2$  months. The VDR expression in OLG, and the striking inverse correlation between circulating 25(OH)D levels and new lesion formation, suggested that 1,25(OH) $_2$ D $_3$ –VDR signaling may protect and restore myelin, and conversely, that hypovitaminosis D may contribute to myelin loss and remyelination failure in the MS brain.

### 6.2 1,25(OH) $_2$ D $_3$ supports the pCD4 $^+$ Foxp3 $^+$ Treg cells that promote myelin repair and replacement

Animal modeling studies tested this hypothesis and began to elucidate the underlying mechanisms. In rodents with EAE disease, 1,25(OH) $_2$ D $_3$  treatment rapidly improved ambulation, decreased brain inflammation, restored myelin architecture, and increased pCD4 $^+$ Foxp3 $^+$  Treg cells [163,254]. These EAE results suggested involvement of pCD4 $^+$ Foxp3 $^+$  Treg cells in the mechanism. Further EAE studies demonstrated 1,25(OH) $_2$ D $_3$ -mediated increases in neural stem cell numbers, immature oligodendrocyte precursor cells (OPC), and mature, myelinating OLGs [255]. More recent EAE data demonstrated that early 1,25(OH) $_2$ D $_3$  treatment controlled neuroinflammation, reduced oxidative stress parameters, preserved myelin, and improved blood–spinal cord barrier function [256]. Research in toxin-based demyelination models showed that 1,25(OH) $_2$ D $_3$  treatment preserved existing myelin and increased new myelin formation [257–259].

New animal modeling studies have probed how pCD4 $^+$ Foxp3 $^+$  Treg cells contribute to neuroprotective mechanisms. As noted before, 1,25(OH) $_2$ D $_3$  drove CD4 $^+$  Th17 cells to transdifferentiate into pCD4 $^+$ Foxp3 $^+$  Treg cells. New data show that CNS-resident pCD4 $^+$ Foxp3 $^+$  Treg cells promoted regenerative functions including remyelination after toxin-induced demyelinating injury [260]. Depleting the pCD4 $^+$ Foxp3 $^+$  Treg cells abrogated OPC differentiation and mature OLG remyelinating activity, and replacing the pCD4 $^+$ Foxp3 $^+$  Treg cells restored this developmental pathway. Moreover, adding pCD4 $^+$ Foxp3 $^+$  Treg cells to murine brain slice cultures before or after toxin-induced demyelination increased the number of mature, myelinating OLG and promoted axon ensheathment. Among the proteins secreted by the pCD4 $^+$ Foxp3 $^+$  Treg cells, CCN3 was indispensable for these beneficial effects. Other work suggested that 1,25(OH) $_2$ D $_3$  increased CCN3 gene transcription in cultured human fibroblasts, but human T cells were not investigated [261]. For

more information on murine pCD4 $^+$ Foxp3 $^+$  Treg cells and CNS remyelination, we refer interested readers to a recent review [156]. In the future, it will be important to probe the relationships between 1,25(OH) $_2$ D $_3$  and human pCD4 $^+$ Foxp3 $^+$  Treg cells and their secreted protein products in the context of myelin repair and replacement by either surviving or new OLG.

### 6.3 VDR signaling enhances oligodendrocyte differentiation and myelin synthesis

The demonstration of high VDR expression in OLG lineage cells, microglia, neurons, and astrocytes in remyelinating MS lesions suggested possible beneficial effects of 1,25(OH) $_2$ D $_3$ –VDR signaling in MS that were not related to immune regulation [262]. Toxin-induced demyelination model systems have been used extensively to probe this possibility, because inflammation does not occur in these models. In one such model, vitamin D–replete animals sustained less axonal damage and loss during the demyelination phase than vitamin D–deficient animals [263]. However, administering 1,25(OH) $_2$ D $_3$  to vitamin D–deficient animals very late in the toxin-induced demyelination/remyelination protocol provided no benefits. During the early remyelination phase, 1,25(OH) $_2$ D $_3$  improved mitochondrial function [264]. Supplementary vitamin D $_3$  promoted neural stem cell proliferation, OPC formation and migration to lesions, and mature, myelinating OLG differentiation within the lesions [265]. Furthermore, antagonizing VDR signaling in rodent cerebellar slice cultures decreased myelination by 52%. Also, antagonizing VDR signaling after toxin-induced demyelination decreased remyelination by 48%. These effects have been attributed to 1,25(OH) $_2$ D $_3$ –VDR signaling in OLG lineage cells independently of immune regulation.

Some controversy surrounds the topic of myelin regeneration in MS patients, specifically, whether it reflects myelin synthesis by surviving, albeit damaged OLG, or by new OLG derived from the OPC pool [266]. There is also debate as to whether remyelination is clinically relevant, since so many axons are lost in the acute inflammatory phase of MS. There is no doubt that myelin repair and replacement is an ongoing process in healthy adults, with OPC differentiation to the mature, myelinating OLG phenotype being a prerequisite [267]. Carbon-dating studies assessed the integration of  $^{14}$ C derived from nuclear testing into OLG genomic DNA and myelin protein in healthy adults [268]. These data showed low-level OLG turnover (0.33%/year) and continuous myelin turnover, supporting the conclusion that new OLGs slowly replace old OLGs in healthy young adults, although this process diminishes with age. The treadmill model of myelin

biosynthesis envisions mature OLGs continuously producing fresh myelin membrane for remodeling, plasticity, and repair [269].

Questions still remain regarding myelin repair and replacement in MS patients. The carbon-dating studies did not detect OLG turnover in MS patients, but there were exceptions. These data were interpreted as a failure of OLG regeneration and myelin remodeling in the MS patients [270]. The data were also interpreted as invalidating the toxin-induced demyelination models, where OLG regeneration and new myelin synthesis after demyelinating injury had been documented. It is important to note the substantial differences inherent in MS and animal modeling research. A limiting factor in MS research is that postmortem samples derive from a single time point in an older individual with late-stage disease, where lesion history or future as regards myelin is uncertain. In contrast, animal modeling provides an opportunity to apply sophisticated gene targeting and lineage tracing tools and precise analytical methods to follow OLG differentiation, myelin sheath formation, and nutritional and microenvironmental influences over time. Some MS patients clearly exhibit remyelination, whereas others do not. This variability likely reflects both genetic and environmental influences. From the vitamin D perspective, it will be important to investigate several parameters that could significantly impact myelin sheath repair and replacement. For example, it would be worthwhile to study the possible influences of sex, vitamin D nutriture (replete vs. insufficient), and the capacity of activated microglial cells to produce  $1,25(\text{OH})_2\text{D}_3$  in the CNS. For a detailed discussion of the controversies surrounding remyelination in MS and the animal models, we refer interested readers to a review [266].

There are many observations that together suggest the hypothesis that  $1,25(\text{OH})_2\text{D}_3$  supports OPC differentiation and mature OLG myelin sheath repair and replacement in RRMS patients. We review three of them here. First, the longitudinal MRI studies summarized before revealed a near perfect inverse correlation (with a 2-month lag) between a period of high ambient solar radiation, a rise to peak  $25(\text{OH})\text{D}$  levels, and the rapid decline of new T2 lesion formation [250–252]. Second, as described before, loss-of-function *CYP27b1* alleles have been associated with MS risk. Third, recent EAE experiments targeted the *Cyp27b1* gene specifically in myeloid lineage cells, including the microglial cells that produce  $1,25(\text{OH})_2\text{D}_3$  in the CNS [13]. This *Cyp27b1* gene targeting completely abrogated vitamin  $\text{D}_3$ -mediated EAE resistance in female mice. This and prior microglial cell studies [164,271] indicate that microglial cell production of  $1,25(\text{OH})_2\text{D}_3$  is essential to protect the CNS from immune-mediated tissue damage. The *Cyp27b1* gene targeting also inhibited CTLA-4

expression by CNS-infiltrating  $\text{CD4}^+$  T cells, which could serve as a critical immunological checkpoint to prevent CNS tissue damage [13]. Juxtaposing these three observations suggests the hypothesis that activated microglial cell  $1,25(\text{OH})_2\text{D}_3$  production and VDR signaling to OPC and OLG promote myelin repair and replacement, and conversely, that hypovitaminosis D contributes to myelin loss and remyelination failure in the MS brain.

#### 6.4 Redox imbalance could undermine 25-hydroxyvitamin D-1 $\alpha$ hydroxylase enzyme activity

These observations raise at least two questions: what signals promote or inhibit microglial cell  $1,25(\text{OH})_2\text{D}_3$  production, and by what mechanisms does VDR signaling promote OLG development for myelin repair and replacement? To the first question, microglial cell *Cyp27b1* gene transcription, extensive experiments have identified the signals that induce nonrenal *Cyp27b1* gene transcription. Those signals include cytokine-mediated immune stimulation, for example, by  $\text{IFN-}\gamma$  [272]. We refer interested readers to Chapter 9.

The question of microglial cell  $1,25(\text{OH})_2\text{D}_3$  production also concerns the enzymatic activity of  $1\alpha$ -hydroxylase, a monooxygenase with a covalently bound cytochrome P450 (CYP450) cofactor. To our knowledge,  $1\alpha$ -hydroxylase enzyme activity in the MS brain has not been adequately examined. However, it is well known that the CYP450 enzymes undergo oxidative damage and inactivation in the context of inflammatory disease [273]. Consequently, there are biochemical reasons to suspect this CYP450 enzyme inactivation rate may be high in the MS brain.

The enzyme's N-terminus is embedded in the inner mitochondrial membrane, where two mitochondrial electron-transport proteins feed  $\text{NADPH}_2$  reducing equivalents via the CYP450 moiety to the substrate in the enzyme active site (see Fig. 101.2 in Ref. [274]). Mitochondrial respiration and the CYP450 catalytic process produce several reactive oxygen species (ROS) including superoxide anion, hydroxyl radicals, singlet oxygen, and peroxides. In MS, profound oxidative damage to nuclear DNA and lipids has been observed mainly in OLG, where damage is associated with evidence of apoptosis [275]. The ROS concentrations are several-fold higher in the mitochondria compared with other subcellular compartments. In inflammatory diseases such as MS, mitochondrial dysfunction increases the rate of ROS generation [276,277]. The ROS molecules oxidize DNA, proteins, and lipids unless they are inactivated enzymatically by reduction. Nitration of Tyr and Cys residues are examples of radical species-induced

protein damage [273]. Many examples of reversible and irreversible CYP450 enzyme inhibition have been reported.

The tripeptide glutathione (GSH; Glu-Cys-Gly) is an important source of reducing equivalents to inactivate ROS and maintain cellular redox balance [278]. However, in the inflamed brain, increased ROS production exceeds GSH-mediated ROS inactivation capacity, leading to excessive oxidative damage to DNA, proteins, and lipids. Protein Cys residues are especially labile to ROS-mediated damage. The  $1\alpha$ -hydroxylase enzyme has 9 Cys residues. One of them, Cys108, covalently binds the CYP450 moiety that is essential for catalytic activity. Another, Cys455, is in the highly conserved Cys pocket, a bulging region that accommodates the bulky CYP450 group (<https://www.uniprot.org/uniprotkb/O15528/entry>). Either S-nitrosation or oxidation ruptured the thiol-CYP450 bond, dissociated the heme, and irreversibly inactivated other enzymes in this class [273]. It is worth noting that other steroid hormone biosynthetic enzymes are also mitochondrial enzymes in the CYP450 family with similarly vulnerable Cys residues [279]. In summary, the inflamed brain is particularly vulnerable to oxidative stress because ROS production exceeds GSH-mediated ROS inactivation capacity, enzymes that produce hormones, maintain redox balance, and control inflammation are easily damaged, and the energy metabolism needed to repair and replace important proteins and structures is reduced [278]. If the  $1\alpha$ -hydroxylase inactivation rate were to be high in the MS brain, then providing supplementary vitamin D<sub>3</sub> to MS patients would not be expected to support OLG myelin repair and replacement, because there would be insufficient active enzyme for 1,25(OH)<sub>2</sub>D<sub>3</sub> biosynthesis.

## 6.5 VDR signaling enhances the methionine cycle, DNA methylation, and oligodendrocyte precursor cell differentiation

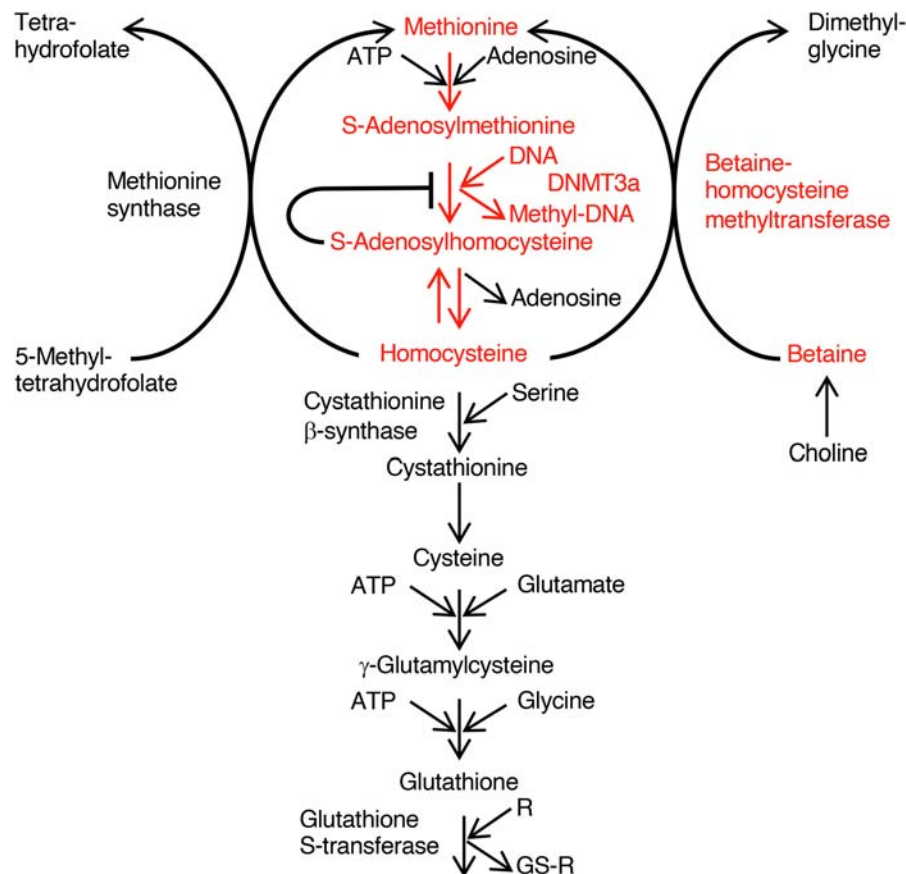
The answer to the second question, what mechanisms allow 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling to promote OLG development for myelin repair and replacement, brings the focus to 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated enhancement of the methionine (MET) cycle (Fig. 101.2). MET, in the form of S-adenosyl-methionine (SAM), provides single carbon units for DNA and protein methylation reactions. DNA methylation is essential to silence the transcriptional repressors that block OLG maturation and myelin synthesis. In the following, we summarize the role of DNA methylation in the OLG developmental pathway and the failure of DNA methylation and OLG development in MS. Then, we review the evidence that nuclear localization of the MET cycle enzyme, betaine-

homocysteine S-methyltransferase (BHMT), is critical for DNA methylation, OLG development, and myelin biosynthesis. Lastly, we review the evidence that 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling enhances *Bhmt* gene transcription, enzyme activity, and DNA methylation in CD4<sup>+</sup> T cells and possibly other cell types [15].

Despite myelin's importance to human evolution, the molecular basis of OLG differentiation and myelin biosynthesis, repair, and replacement functions has only recently been elucidated [267]. It is now clear that complex extracellular signals, intracellular transcriptional networks, and epigenetic mechanisms guide the slow, dynamic process of OPC lineage specification in neural stem cell niches, OPC migration to exposed axons, and OPC terminal differentiation into myelinating OLG. The discovery of transcriptional repressors that maintain OPC in an immature, proliferating state, and transcriptional enhancers that promote OLG expression of essential genes for myelin protein and lipid biosynthesis has been an important achievement. The OPCs that are observed in demyelinated, nonfibrotic MS lesions appear to be blocked from differentiating into mature OLG capable of repairing and/or replacing damaged myelin [280]. As MS disease progresses toward a chronic stage, myelin biosynthesis, repair, and replacement activity gradually become insufficient to maintain myelin sheaths.

This past decade has witnessed a growing appreciation for DNA methylation's involvement in human development and disease generally [281], and in MS specifically [282]. Recent research probing the biochemical basis for blocked OPC differentiation in MS has focused on DNA methylation of the transcriptional repressors that maintain immature OPC in a proliferating state [282]. In the toxin-induced demyelinating model, OLG-specific targeting of DNA methyltransferase genes 1 (*Dnmt1*) and 3a (*Dnmt3a*) decreased remyelinating OLG cell numbers and remyelination efficiency [283]. Two genes encoding transcriptional repressors of OLG development, inhibitor of differentiation-2 (*Id2*) and inhibitor of differentiation-4 (*Id4*), proved to be DNA methylation targets [267]. These gene promoters were hypermethylated and silent in mature OLG. However, blocking DNA methylation induced their expression and prevented mature OLG development, while targeted hypermethylation of these gene promoters silenced transcription and promoted mature OLG differentiation. Thus, *Id2/Id4* promoter methylation was essential for OLG differentiation and myelin protein gene expression in this demyelinating model.

Extending the analysis to humans, the *ID2/ID4* promoter methylation status and mRNA expression were analyzed in postmortem brain tissue samples from MS normal-appearing white matter, MS lesions, and healthy



**FIGURE 101.2 Betaine–homocysteine methyltransferase, the methionine–homocysteine cycle, and DNA methylation.** The central circle red text describes the addition of adenosine to methionine (MET) by methionine adenosyl-transferase to form S-adenosyl-methionine (SAM), the universal methyl donor for all methylation reactions. In the nucleus, SAM is consumed by methylating enzymes like DNA methyltransferase 3a (DNMT3a), which adds methyl groups to particular cytosine residues within CpG clusters. Other methyltransferase enzymes consume SAM to add methyl groups to proteins such as histones and myelin basic protein. Adenosyl homocysteinase reversibly hydrolyzes the methylation reaction product, S-adenosylhomocysteine (SAH), to homocysteine (HCY) and adenosine. If SAH is not hydrolyzed and the HCY removed, the SAH remains bound to the methyltransferase enzyme active site as an end product inhibitor. The HCY can be recycled to MET by two distinct pathways. The most familiar pathway, shown as the half circle on the left, involves 5-methyltetrahydrofolate–homocysteine methyltransferase (MTR), also known as methionine synthase, which transfers a methyl group from 5-methyltetrahydrofolate to HCY. The relatively unknown pathway, shown as the half circle on the right, involves betaine–homocysteine methyltransferase (BHMT), which transfers a methyl group from betaine to HCY. The HCY can also be removed by the ATP-dependent, transsulfuration pathway shown below the central circle. This pathway ultimately yields glutathione (GSH), an important reducing agent for inactivation of cytosolic reactive oxygen species (ROS) generated by mitochondrial respiration.

control white matter [267]. Strikingly, the MS tissue samples exhibited low *ID2/ID4* gene promoter methylation, high *ID2/ID4* gene transcription, and low myelin protein gene expression compared with healthy controls. Thus, incomplete DNA methylation of the repressive *ID2/ID4* genes correlated with a failure of OLG myelin biosynthesis in MS lesions and normal-appearing white matter. These experiments raise critically important questions: what controls DNMT enzyme activity and DNA methylation of repressive genes in the OLG developmental pathway, and why is this activity suboptimal in MS?

## 6.6 1,25(OH)<sub>2</sub>D<sub>3</sub>, the BHMT gene, betaine, and methionine cycle enhancement

One straightforward answer to the first question is that DNMT enzyme activity reflects the availability of its substrate, SAM (Fig. 101.2). SAM is the methyl donor for all methyl group transfer reactions [284]. All methylation reactions generate S-adenosylhomocysteine (SAH) as a reaction product. The SAH remains bound to the enzyme active site as an inhibitor unless it is hydrolyzed to homocysteine (HCY). The HCY can be recycled to MET and then to SAM by the MET cycle. Alternatively,



HCY can be metabolized to GSH by the energy-dependent, transsulfuration pathway. As noted before, GSH is especially important in the brain, where its depletion is associated with many neurodegenerative diseases [278].

If HCY is not recycled to MET or metabolized to GSH, it can be released to the circulation where it is a highly toxic metabolite. Many studies have reported elevated circulating HCY levels in MS patients, particularly in men [16,285–287]. Elevated circulating HCY correlated with MS disease progression [287], cognitive impairment [288–290], depression [291], and brain atrophy [292]. These findings prompted the hypothesis that MS patients may have a defect at some point in the MET cycle, and the resulting elevated circulating HCY levels might contribute to MS by an as-yet-unidentified mechanism. A causal link between elevated circulating HCY levels and damage to the vasculature including the cerebral vasculature has been demonstrated [293]. However, it is not yet clear whether a defect in the MET cycle and elevated circulating HCY levels contribute causally to MS risk or pathogenesis.

The MET cycle offers two biochemical routes from HCY to MET (Fig. 101.2). A well-known route begins with the conversion of dietary folate to N,N-methylene-tetrahydrofolate, and the reduction of this metabolite to N-methyl-tetrahydrofolate by the enzyme N,N-methylene-tetrahydrofolate reductase (MTHFR). Subsequently, 5-methyltetrahydrofolate–homocysteine methyltransferase (MTR; also known as methionine synthase) transfers the N-methyl group from N-methyltetrahydrofolate to HCY, regenerating MET. The MTHFR enzyme, folate, and the cofactor cobalamin have been extensively studied in the context of neural tube birth defects [294].

Less well known is the other biochemical route from HCY to MET, beginning with dietary betaine or choline (which is converted into betaine). The zinc-dependent BHMT enzyme transfers a methyl group from betaine to HCY, regenerating MET. For many years, BHMT was thought to be present only in the liver and the kidney [295,296]. Moreover, since BHMT exhibits rather poor binding constants for its substrates [297], it was believed to be only a minor contributor to the HCY recycling process. However, the observation that BHMT was present and conserved in the genomes of the sea urchin, amphibians, reptiles, birds, and mammals suggested it performed some essential metabolic role [298]. Exciting new evidence has linked nuclear BHMT enzyme expression with DNA methylation and epigenetic silencing of repressive genes in the OLG developmental pathway [17]. Other new evidence demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling promoted *Bhmt* gene transcription in CD4<sup>+</sup> T cells, sparing rodents from developing hyperhomocysteinemia and EAE disease [15]. In

combination, these two important advances suggest the novel hypothesis that 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling supports the function of a nuclear betaine–MET pathway involved in methylation reactions that are critical for epigenetic regulation of gene expression in cells relevant to MS.

Pioneering animal modeling experiments led to the discovery of new biological roles for the betaine–MET cycle. Firstly, recent experiments targeting the *Bhmt* gene in mice found that animals lacking a functional *Bhmt* gene had toxic circulating HCY levels, reduced brain volume, and impaired memory formation [299]. Secondly, other experiments revealed that providing supplementary anhydrous betaine inhibited EAE [300]. Thirdly, betaine, given prior to toxin-induced demyelination, improved brain SAM levels, restored nuclear epigenetic marks, enhanced respiration, and prevented axonal damage in rodents [301]. Fourthly, BHMT protein was identified in the cytoplasm and the nucleus of immature OPC and mature, myelinating OLG in rodent cell and tissue samples [17]. The BHMT, DNMT3a, and histone methyltransferase were in a multi-protein complex bound to DNA.

Human OPC cultures were used to test the functionality of the BHMT–DNMT3a protein complex [17]. The OPCs were chemically treated to induce oxidative damage in the presence or absence of added betaine. The betaine addition increased the stability of the BHMT protein and stimulated OPC differentiation to the mature, myelinating OPG phenotype. Further experiments employed siRNA knockdown of *Bhmt* mRNA in the OPC, followed by culture in the presence or absence of added betaine. Betaine addition increased DNMT3a enzyme activity in control cells, but not in cells with siRNA knockdown of *Bhmt*. Extending their analysis to postmortem MS brain tissue, these investigators detected nuclear BHMT staining in oligodendrocyte lineage cells, possibly OPC, within lesioned areas. These and other data [302] established the functional importance of betaine and BHMT in complex with DNMT3a in the OPC nucleus. In this position, the BHMT could funnel SAM directly to DNMT3a for epigenetic marking. Collectively, these remarkable human and animal results point to a deficiency of betaine and the BHMT enzyme, as well as MET cycle dysfunction, as possible contributing factors in MS pathogenesis. Conversely, the data suggest the exciting possibility that providing betaine and increasing BHMT enzyme expression might improve the MET cycle, restore redox balance, increase respiration, and promote OLG differentiation for myelin repair and replacement in MS patients.

The question arises whether 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling would increase *Bhmt* gene transcription, BHMT enzyme activity, and DNA methylation in CNS-resident cells like OPC and OLG, as it did in murine

CD4<sup>+</sup> T cells. Recent experiments demonstrated that one 1,25(OH)<sub>2</sub>D<sub>3</sub> dose plus supplementary vitamin D<sub>3</sub> reversed EAE disease in mice with transgenic CD4<sup>+</sup> T cells specific for an MBP epitope [15]. This protocol rapidly increased *Bhmt* transcript abundance ~1.6-fold, BHMT enzyme activity ~3-fold, and global DNA methylation in the transgenic CD4<sup>+</sup> T cells. It also increased *Bhmt* transcript abundance in kidney cells. In cultured transgenic CD4<sup>+</sup> T cells, adding 5-aza-deoxycytidine blocked the stimulating effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on global DNA methylation. These data indicate that a T cell-intrinsic process was responsible for installing methyl marks on new DNA.

Follow-up studies in mice with CD4<sup>+</sup> T cell-specific *Vdr* gene targeting revealed sharply decreased *Bhmt* transcript abundance and BHMT enzyme activity in the *Vdr*-targeted CD4<sup>+</sup> T cells [15]. Unexpectedly, mice with CD4<sup>+</sup> T cell-specific *Vdr* gene targeting and EAE disease had elevated circulating HCY levels. Additional experiments established that CD4<sup>+</sup> T cells did not express the *Mtr* gene encoding methionine synthase. A search algorithm identified a sequence, 5'AGGCCAAGAAGGTGA, with strong homology to DR3-type VDREs that is 100% conserved in the mouse *Bhmt* and human *BHMT* promoter regions. Collectively, these surprising observations indicate that proliferating, myelin-specific CD4<sup>+</sup> T cells must rely on the BHMT–betaine pathway to recycle HCY into MET, since they have no MET synthase–methyl–tetrahydrofolate pathway (Fig. 101.2). When these CD4<sup>+</sup> T cells had no functional VDR, they also lacked the BHMT–betaine pathway. So, they released toxic levels of HCY into the circulation because they could not recycle it.

In summary, new pioneering experiments have greatly advanced our mechanistic knowledge of the vitamin D system's supportive roles in OLG differentiation and myelin synthesis. A direct mechanism has come into focus reflecting 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated enhancement of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation and tissue homeostatic functions that promote myelin repair and replacement. A second novel mechanism reflects 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling directly in OLG lineage cells independently of immune regulation. Vitamin D–replete animals exhibited improved mitochondrial function, neural stem cell proliferation, OPC formation and migration to lesions, and mature, myelinating OLG within the lesions, whereas vitamin D–deficient animals did not. Further animal experiments demonstrated that promoter DNA methylation of two transcriptional repressors, *Id2/Id4*, allowed rodent OPC to differentiate into myelin-forming OLG. In postmortem MS tissue samples, hypomethylation of the *ID2/ID4* gene promoters correlated with high *ID2/ID4* gene transcription and the absence of OLG expressing *MBP* gene transcripts.

Additional animal experiments revealed a protein complex of BHMT and DNMT3a in the OPC nucleus. This complex transferred single carbon units from betaine to SAM for DNA epigenetic marking. This discovery suggests a novel role for the betaine–MET cycle in epigenetic regulation of gene expression. Finally, research in the EAE model indicated 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signaling enhanced *Bhmt* gene expression, MET cycle function, DNA methylation, and encephalitogenic CD4<sup>+</sup> T cell transdifferentiation into pCD4<sup>+</sup> Treg cells. In combination, these advances suggest the hypothesis that activated microglial cell 1,25(OH)<sub>2</sub>D<sub>3</sub> production and VDR signaling to OPC may promote OLG differentiation for myelin repair and replacement, and conversely, hypovitaminosis D and lack of liganded VDR signaling may contribute to myelin loss and remyelination failure in the MS brain.

## 7. Some reflections on vitamin D<sub>3</sub> supplementation trials in MS

Past clinical trials in MS patients have demonstrated mixed outcomes [303,304]. Those studies have sometimes failed to consider details regarding randomized matching of the treatment and control groups, vitamin D status at enrollment, supplementary vitamin tablet use, vitamin D<sub>2</sub> versus vitamin D<sub>3</sub>, safe and effective vitamin D supplementation protocols, metabolite analytical challenges, power calculations, and many other important aspects of human clinical trial design. Nevertheless, many reports do suggest that vitamin D<sub>3</sub> supplementation promoted anti-inflammatory and regulatory immune parameters, while reducing proinflammatory parameters.

The earliest report, a dose–response study, tested the safety of 4000 to 40,000 IU/day for 28 weeks in MS patients [305]. Circulating 25(OH)D levels in some patients reached twofold higher than the physiologic range with no evidence of hypercalcemia or hypercalciuria, whereas other patients exhibited only a limited increase in this metabolite. The authors concluded that 10,000 IU/day was “safe by a large margin.” Fifteen years later, it is now clear that circulating 25(OH)D levels are under genetic control (see Chapter 60), and some of these genetic variants are risk factors for MS (see previous discussion). New insights might be gained in forthcoming vitamin D supplementation trial designs if baseline and longitudinal 25(OH)D levels were to be measured, if enrollment were to be limited to MS patients with hypovitaminosis D, and if genetic variants that regulate circulating 25(OH)D levels were to be investigated in MS patients who do not exhibit the anticipated increase in circulating 25(OH)D.

There has been considerable debate regarding the thresholds for vitamin D deficiency, insufficiency, and adequacy. Defining optimal 25(OH)D levels from the nutritional perspective has proven to be difficult, but defining this parameter biochemically is straightforward [306]. The vitamin D-25-hydroxylase responds to a broad range of vitamin D<sub>3</sub> concentrations in a biphasic manner. When vitamin D concentrations are low, the 25-hydroxylase produces 25(OH)D rapidly with first-order reaction kinetics, indicating the supply of substrate does not saturate the available enzyme. However, when vitamin D<sub>3</sub> is >15 nmol/L and 25(OH)D is >80 nmol/L, the enzyme produces 25(OH)D slowly with zero-order reaction kinetics. These data indicate that the product, 25(OH)D at >80 nmol/L, is impeding the enzyme active site. Interestingly, evolution appears to have optimized human biology to function with 80–115 nmol/L of 25(OH)D as the physiological level [307]. Consequently, 80–115 nmol/L of 25(OH)D is considered by many, including us, to be optimal for human health.

There has also been controversy regarding how to achieve adequate circulating 25(OH)D levels in individuals and populations. Given individual genetic variations that influence circulating 25(OH)D levels, it is difficult to make a recommendation at the population level. For this reason, some experts now advocate for the concept of a personalized vitamin D response index to assess supplementation efficacy [308]. A thorough critique of this complicated topic is beyond the scope of this chapter. Instead, we refer interested readers to Chapter 57, and to the following reviews for precise details in the MS field [309,310]. It is imperative to note that life-threatening vitamin D intoxication in humans can result from ingestion of extremely high vitamin D<sub>3</sub> doses, for example, >100,000 IU/day, due to untested, unconventional protocols for MS treatment [311].

Investigators anticipate that the results of ongoing trials with earlier interventions and more robust clinical trial designs may shed light on the actual role of vitamin D<sub>3</sub> supplementation in delaying or preventing an MS diagnosis. One ongoing trial is the D-Lay trial (*trial NCT01817166; D-Lay-MS - Efficacy of Cholecalciferol (Vitamin D3) for Delaying the Diagnosis of MS After a Clinically Isolated Syndrome*), testing supplementation in French patients with clinically isolated syndrome. Clinically isolated syndrome refers to individuals who have experienced a first episode of neurological disability caused by inflammatory demyelination; formal diagnosis of MS requires evidence of multiple attacks, affecting distinct portions of the central nervous system. Evidence for such dissemination in space and time can come from clinical relapses or from specific MRI changes. CSF-specific oligoclonal bands can serve as a substitute for dissemination in time [312]. The D-Lay

trial aims to provide a placebo or 100,000 IU of supplementary vitamin D<sub>3</sub> every 14 days (7143 IU/day) for a maximum of 2 years to clinically isolated syndrome patients. The D-Lay trial results may provide a clearer picture of the effects of vitamin D<sub>3</sub>, since patients do not have ongoing immunomodulatory treatments, which may confound or bias the results.

## 8. Conclusion

**Summation.** MS has a long history. In 1757, the first reasonably convincing case of MS was reported in the medical literature [1]. In 1960, researchers wrote “... the more sunshine there is in a climate, the less multiple sclerosis there appears to be” [18]. In 2023, we write “vitamin D insufficiency is now believed to contribute causally to MS pathogenesis.” This conclusion is based on compelling evidence gathered from experiments in genetics, biochemistry, immunology, nutrition, and medical science in just the past 5 years. Several research advances stand out. Mendelian randomization studies now confirm genetically determined low serum 25(OH)D as an MS risk factor. Immunological research has highlighted cross-talk between the vitamin D system and the complement regulator CD46 controlling the transdifferentiation of proinflammatory CD4<sup>+</sup> Th1 cells to regulatory CD4<sup>+</sup> Tr1 cells, and the transcription factor FOXP3 controlling the transdifferentiation of proinflammatory CD4<sup>+</sup> Th17 cells to regulatory pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cells. Both regulatory CD4<sup>+</sup> T cell subsets prevent immune-mediated tissue damage, and both are defective in MS. Biochemical evidence has implicated myeloid lineage cells as the producers of 1,25(OH)<sub>2</sub>D<sub>3</sub> for highly localized signaling to VDR-expressing in the CNS and lymphoid tissues and suggested a model for direct VDR-mediated control of MS-relevant gene transcription. Other biochemical evidence has highlighted cross-talk between the vitamin D system and the MET cycle that supplies single carbon units for epigenetic marking and suggested a model for indirect VDR-mediated control of MS-relevant gene transcription through DNA or histone methylation in OPC differentiation. A transgender study reported the sex hormones modulated MS risk independently of the sex chromosomes and suggested a hypothetical model to explain MS sexual dysmorphisms through 1,25(OH)<sub>2</sub>D<sub>3</sub> and sex hormone interactions in FOXP3 regulation. Now, 265 years after the first probable case of MS was reported, we can describe in some detail a pathway that begins with cutaneous exposure to solar radiation and ends with engagement of the 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR complex with nuclear DNA in several types of MS-relevant cells where it triggers protective mechanisms that together reduce the risk of MS.

**Importance.** MS remains incurable. The global MS disease incidence has increased nearly fivefold in just six decades. MS afflicts nearly 3 million people, most of them women. Many of them are young, productive adults. Scientists have long believed that modifiable environmental risk factors interact with genetic risk factors and sex hormones to cause the demyelination and focal inflammatory responses that are observed in MS. We now know that severe hypovitaminosis D appears to be the strongest modifiable environmental risk factor for MS, with the relative MS risk increasing >10-fold as circulating 25(OH)D levels decline from ~110 to <10 nmol/L (see Fig. 10.4C in Ref. [7]). We have detailed knowledge of genetic risk factors and cells of interest. Oligodendrocytes hold the key to myelination, and CD4<sup>+</sup> T cells hold the key to immunoregulation. We understand the mechanisms of 1,25(OH)<sub>2</sub>D<sub>3</sub> and sex hormone actions, and some target genes have been identified. Two published algorithms predicted MS genetic risk with some success. A report detailing DNA methylation quantitative trait loci in CD4<sup>+</sup> T cells may serve as a guide to find accessible biomarkers of regulatory CD4<sup>+</sup> T cell function in individuals with a predicted high risk of MS. Lastly, we understand how to address nutritional vitamin D insufficiency. Because of these

recent and remarkable scientific advances, the vitamin D and MS research community is poised to modify nutritional vitamin D insufficiency in individuals with a predicted high genetic risk of MS, and quite possibly alter the trajectory to an MS diagnosis. What a remarkable accomplishment that would be.

**Future.** We finished each section of this chapter with open questions for continued research. In Box 101.2, we summarized the knowledge gaps we identified in genetics, immunology, biochemistry, nutrition, and medical science and made a few suggestions for future research. This list of questions and suggestions is not complete, but we hope it will serve as an inducement to advance vitamin D and MS research as rapidly as possible. The results of better-controlled vitamin D<sub>3</sub> intervention trials in individuals with clinically isolated syndrome and hypovitaminosis D, coupled with longitudinal measurement of biomarkers and clinical data, and the deep phenotyping or sequencing analyses of relevant cells donated by participants will very likely increase our insights into mechanisms that apply after the disease process has begun. However, the important long-range goal is MS prevention. Once we understand how to identify individuals at risk of developing MS with precision, and how to modify the strongest of

### BOX 101.2

#### Research challenges from the vitamin D and MS perspective.

##### Challenges in genetics

The MS genetic risk variant-to-function problem remains a major focus. Identifying the downstream transcriptional mechanisms that transduce MS genetic risk and are subject to VDR regulation in MS-relevant cells will be important. A first step will be discovering MS-relevant genes that are differentially expressed in key cells (e.g., monocytes, CD4<sup>+</sup> T cells, or OPC) in the vitamin D–replete and vitamin D–deficient states. A second step will be determining which transcriptional regulatory processes (e.g., chromatin accessibility, epigenetic marking, DNA looping, transcription factor binding, transcription complex recruitment) are influenced either directly or indirectly by the liganded VDR. Also important will be determining how microenvironmental conditions (e.g., sex hormones, inflammatory signals) influence the transcriptional regulatory processes. Of high interest are epigenetic marks in *HLA-DRB1* that have been associated with MS, particularly marks associated with the *DRB1\*1501* risk allele. Would the vitamin D–replete and vitamin D–deficient states influence these epigenetic marks and MS risk gene expression? Also of high interest

are the hormone-responsive enhancers in the *FOXP3* gene that have been associated with extrathymic pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation and function. Could differential 1,25(OH)<sub>2</sub>D<sub>3</sub>, TST, and E2 enhancement of *FOXP3* gene transcription in males and females be a mechanism linking declining 25(OH)D status with increasing MS risk in a female-biased manner?

##### Challenges in biochemistry

In the vitamin D–replete state, activated microglial cells produced 1,25(OH)<sub>2</sub>D<sub>3</sub>, signaling to diverse cells within the CNS to protect this tissue from autoimmune damage. However, it is unclear why vitamin D<sub>3</sub> repletion has not benefitted patients with MS or animals with EAE. Could the rate-limiting 1 $\alpha$ -hydroxylase for 1,25(OH)<sub>2</sub>D<sub>3</sub> biosynthesis be inactivated under inflammatory conditions? For example, if ROS production were to exceed ROS inactivation capacity in the inflamed brain, would ROS-mediated damage irreversibly inactivate the 1 $\alpha$ -hydroxylase?

The ligand-activated VDR enhanced *Blmt* gene expression and the betaine–MET cycle in murine CD4<sup>+</sup> T cells.

*Continued*



### BOX 101.2 (cont'd)

Does this also occur in other cells such as human CD4<sup>+</sup> T cells or OPC? The BHMT enzyme was bound to DNMT3a in the OPC nucleus where the complex enhanced DNA methylation when betaine was added. Does the BHMT–DNMT3a complex enhance DNA methylation in a VDR-dependent manner in OPC? Could this be a repressive epigenetic mechanism controlling expression of the *ID2/ID4* genes in OPC or the *DRB1\*1501* allele in antigen-presenting cells?

#### Challenges in immunology

VDR signaling dampened neuroinflammation, reduced oxidative stress parameters, and increased myelin synthesis through its actions on rodent OPC and pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells. Do these observations apply to human OPC and pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells and to oxidative stress in the human? What CNS tissue homeostatic functions of pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells can be increased by 1,25(OH)<sub>2</sub>D<sub>3</sub>–VDR signals? How do sex hormones influence pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells? What is the impact of VDR signaling on myelin repair and replacement by either surviving or new OLG in the human? Previous data indicate that *CTLA4* inactivating mutations cause an IPEX-like disease in humans, and new rodent data point to direct VDR-mediated upregulation of CTLA-4 expression in CD4<sup>+</sup> T cells. Do the vitamin D–replete and vitamin D–deficient states influence the expression and/or function of this critically important immune checkpoint molecule in humans?

#### Challenges in nutrition

Serial MRI of RRMS patients revealed a seasonal periodicity with decreases in ambient UV radiation and circulating 25(OH)D preceding changes in new T2 lesion formation by ~2 months. Does this observation apply

only in high-latitude regions, or also in low-latitude regions? Does the patient's age, sex, or vitamin D nutriture (replete vs. insufficient) influence the seasonal periodicity of new T2 lesion formation?

Many studies have reported elevated circulating HCY levels in MS patients, prompting the hypothesis that these patients may have a dysregulated MET cycle, which could affect the cerebral vasculature, redox balance, and epigenetic marking within cells. How common is elevated circulating HCY in MS patients? Would nutritional intervention to correct elevated circulating HCY levels benefit MS patients with this condition? The MS risk in transsexual females was 4.6-fold higher than the risk in transsexual males in a small pilot study. Is this a reproducible finding? Does vitamin D nutriture (replete vs. insufficient) have an influence on MS risk in this population?

#### Challenges in healthcare

Investigators anticipate that the results of ongoing trials with earlier interventions and more robust clinical trial designs may shed light on the actual role of vitamin D<sub>3</sub> supplementation in delaying or preventing an MS diagnosis. Can the harvest of information from clinical trials be improved by longitudinal monitoring of 25(OH)D as well as biomarkers related to CD4<sup>+</sup> Treg cell and CD4<sup>+</sup> Tr1 cell function and MET cycle function?

At least two MS risk prediction algorithms have been published [195,313]. What accessible biomarkers might forecast a prodromal period for MS disease, wherein dysregulated biological processes may be underway but even subtle signs of neurological disability are not yet detectable? Could epigenetic marks in key regions of MS risk genes within MS-relevant cells forecast a dysregulated immune response and suggest new approaches to reduce the risk of an MS diagnosis?

the environmental risk factors, vitamin D insufficiency, we hope millions of people worldwide might be spared an MS diagnosis.

### 9. Summary points

- Observational epidemiology provides strong evidence that low vitamin D status increases the risk of MS and serum 25(OH)D is genetically influenced;
- Mendelian randomization studies confirm genetically determined serum 25(OH)D as one of the causes of MS; the ligand-activated VDR is involved at every step in the epigenetic regulation of genes (e.g., *HLA-DRB1*, *FOXP3*, *IKZF2*, *CTLA4*, *IL10*) with high relevance to MS, from chromatin opening and recruitment of pioneer factors through direct transcriptional regulation.
- New insights into MS etiological mechanisms are derived from analysis of global longitudinal F:M

incidence data, and F:M incidence data in transsexual individuals; the evidence suggests a hormonally triggered and reversible protective mechanism intrinsic to CD4<sup>+</sup> T cells and linked to reproductive success reduces MS risk and severity.

- The 1,25(OH)<sub>2</sub>D<sub>3</sub> and sex hormone–gene interactions apparently converge to enhance *FOXP3* gene expression and protective pCD4<sup>+</sup>Foxp3<sup>+</sup> Treg cell differentiation and function in a sex-biased manner.
- The interplay between VDR–1,25(OH)<sub>2</sub>D<sub>3</sub> and CD4<sup>+</sup> Th1 cells results in amplification of the CD46 costimulatory signals that promote CD4<sup>+</sup> Th1 cell transdifferentiation to the protective IL-10-producing CD4<sup>+</sup> Tr1 cell phenotype; recent data suggest that the CD4<sup>+</sup> Th1 cells from MS patients and COVID-19 patients might be incapable of undergoing this CD46-mediated switch for reasons that are not yet clear, but may reflect low vitamin D status; these data open new avenues of investigation to improve CD4<sup>+</sup> Tr1 cell differentiation and function in MS patients and COVID-19 patients.
- The discovery of 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated enhancement of *Bhmt* gene expression, the methionine cycle, and DNA methylation in CD4<sup>+</sup> T cells, as well as the discovery of BHMT protein bound to DNA methyltransferases and DNA in the OPC nucleus, suggest an epigenetic hypothesis for 1,25(OH)<sub>2</sub>D<sub>3</sub> support of myelin sheath repair and replacement; in this model, 1,25(OH)<sub>2</sub>D<sub>3</sub> enhances the methionine cycle, which facilitates epigenetic silencing of genes encoding transcriptional repressors of OLG differentiation, thereby promoting myelin repair, replacement, and redox balance in the CNS.

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# Vitamin D and the epidemiology of multiple sclerosis

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## OBJECTIVES

- Provide an overview of multiple sclerosis and descriptive epidemiology.
- Discuss MS epidemiology in the context of vitamin D nutrition as a risk factor for MS.
- Present and discuss the literature on vitamin D and MS risk, including studies of sun exposure, dietary vitamin D, and blood 25-hydroxyvitamin D levels.
- Review vitamin D gene-MS association studies.
- Present and discuss the literature on vitamin D and MS disease activity and progression, including observational studies and randomized controlled clinical trials.
- Examine the evidence that vitamin D nutrition is also a risk factor for pediatric onset MS.

## 1. Introduction

Multiple sclerosis (MS) is a disease of the central nervous system (CNS) characterized by an immune system-mediated breakdown of the myelin sheaths that surround the axons of the nerve cells. Two of the major functions of myelin are to facilitate the rapid conduction of nerve cell signals and to protect the axon from damage. In MS, both roles are compromised

and depending on where in the brain or spinal cord these areas of demyelination (lesions) occur, there is a constellation of symptoms that MS patients may experience including optic neuritis, clumsiness, loss of balance, gait ataxia, and paresthesia in the extremities and trunk to name a few [1,2]. Further, many MS patients also have some degree of debilitating nerve pain, fatigue, and cognitive dysfunction, especially as the disease progresses over time [1]. Over 90% of patients show onset with a relapsing-remitting (RRMS) course in which they experience episodes of neurologic deficit (relapses) followed by periods of complete or incomplete recovery (remittance). The clinical diagnosis of MS requires two episodes of neurologic deficit lasting at least 24 h and occurring at least 30 days apart. When only one episode has occurred, the patient is diagnosed with the clinically isolated syndrome (CIS). CIS is typically an early clinical manifestation of MS as approximately 70%–80% of individuals with a CIS will eventually meet the diagnostic criteria for MS [3,4] either by experiencing a second relapse or exhibiting changes in and developments of CNS demyelination or presence of oligoclonal bands in the cerebral spinal fluid [5]. During the early phases of RRMS, there is both clinical and sub-clinical active inflammation and damage occurring in the CNS [6]. Many people with RRMS will enter a secondary progressive phase (SPMS) some decades after initial symptom onset in which they have an accrual of a permanent neurologic deficit as axonal destruction continues. In addition to the physical disabilities, as MS progresses, brain volume decreases and cognitive

function further declines, drastically affecting the quality of life. A small percentage of MS cases ( $\sim 10\%$ ) onset with a primary progressive disease course in which there is an accumulation of physical and cognitive disabilities over time with no clear relapses or remissions. While innovations in disease-modifying therapies have been beneficial in reducing relapse rates and lesion development in early RRMS, most patients will still progress to SPMS. MS remains an incurable progressive disease with an uncertain etiology and contributors to disease activity and progression. There is very strong evidence for a causal role of infection with Epstein–Barr virus (EBV) [7] with risk further modified by genetics (in particular the human-leukocyte antigen (HLA)-DR15\*01 haplotype), cigarette smoking, overweight/obesity in early life, and insufficient vitamin D nutrition [8]. There is also evidence supporting the beneficial role of adequate vitamin D nutrition in MS disease activity and progression. This chapter will discuss the epidemiology of MS with respect to vitamin D and evidence of vitamin D nutrition as a risk factor for developing MS as well as for MS disease activity and progression. A more detailed description of the immunomodulatory effects of vitamin D in MS is provided in Chapter 101.

## 2. Descriptive epidemiology

MS was first formally named and described by Jean-Martin Charcot in the 1860s. He noted that MS typically onsets in young adults between ages 25–30 years and that women were more likely to be affected than men [9]. Over 150 years later both observations persist. MS is the most common non-traumatic neurologically disabling disease in young adults with a peak incidence of onset around 30–35 years of age [10–12], and an estimated prevalence of 1 million in the US and 2.8 million worldwide [13,14]. The overall incidence of MS has been increasing in many countries and an increase among women specifically appears to be driving this trend [15]. In general, women are 2–3 times more likely to develop MS as compared to men [10–12], but studies in several countries have seen a sharp increase in the female to male ratio over the past 50 or so years [16–19]. Studies in the mid-1900s reported lower incidence of MS among non-Hispanic Black (NHB) women and NHB men as compared to non-Hispanic White (NHW) women and NHW men, respectively [20]. However, more recent studies find that MS incidence and prevalence have increased in both NHB women and men and are now more similar to NHWs [21–23]. The reasons behind the increasing female to male sex ratio and increasing incidence in NHB populations are not understood, but changes in various behaviors such as cigarette smoking and sun exposure may play a role [8].

There have been over 200 genes—the majority involved in immune system function—that have been associated with MS though individual gene contributions to risk are small, and having a first-degree relative with MS is one of the strongest risk factors for the disease [24]. The strongest genetic association for MS is the HLA DRB1\*1501 haplotype; carriers have a threefold increased risk of MS [25]. Thus, MS is a multifactorial disease with risk modification occurring in genetically susceptible individuals.

Two observations that strongly suggested that environmental risk factors contribute to MS are 1) the existence of a latitude gradient in MS prevalence [26,27] and 2) the changing MS risk observed with migration [28–30]. Countries farther away from the equator (i.e., at higher latitudes) tend to have a higher prevalence of MS and this prevalence decreases with decreasing latitude (i.e., moving toward the equator). Though an attenuation of the latitude gradient over the last half-century has been observed in the US [22,27], it continues to persist on a global scale [26,31]. Migration studies both within [32] and between countries [33] generally find that groups who migrate to more northern areas have a higher risk of MS than that in the location they migrated from and those who migrate from northern to southern locations have a lower risk than their original location. Younger age at migration may also be an important factor as some studies suggested that migrants less than 15 years old adopted the MS risk associated with their new location while older migrants did not; [28] however, more recent studies support similar change in MS risk even after the age of 15 [30,33,34]. Both the latitude gradient and migration studies support the existence of an environmental risk factor, and these observations are consistent with the role of vitamin D in determining MS risk given the inverse association between vitamin D nutrition and latitude.

## 3. Vitamin D and MS—historical perspective

Vitamin D was first suggested to be a possible risk factor for MS in the 1970's based on the existence of the latitude gradient, the observation that populations which consumed vitamin D-rich foods (e.g., salmon and other fatty fish) had lower incidence rates of MS as compared to populations with diets low in these foods, and inverse correlations between ecologic measures of average annual sunshine and temperature with MS prevalence [35–37]. Though it would be another 30 years before the first epidemiologic studies linked vitamin D nutrition with MS, there were studies in experimental autoimmune encephalomyelitis (EAE), one animal model of MS, showing that mice supplemented with vitamin D [38–40] or exposed to high

UVR [41] were less likely to develop severe EAE or show no symptoms of EAE at all. These observations, coupled with those that vitamin D has immunomodulatory effects [42], prompted the study of vitamin D nutrition as a potentially important factor in MS development.

## 4. Vitamin D and risk of MS

### 4.1 Sun exposure and MS risk

Given both the latitude gradient for MS and that sun exposure is the primary source of vitamin D, time spent exposed to the sun has been studied as both a proxy measure for vitamin D and as a possible exposure having effects independent of vitamin D through other immune modifying pathways [43]. The earliest studies to include an investigation of the association between sun exposure and MS risk were inconsistent with the higher sun exposure-lower MS risk hypothesis. A study with 300 MS cases and 300 controls with sciatica found no difference in the amount of time spent in the summer sun at age 15 [44], and a study in Israel found that MS patients were more likely to report having spent more than 2 hours per day outside during the summer up to age 15 years [45]. Selection bias is the likely explanation for the results in both studies as the controls used were not representative of the source populations for the cases [8]. Since 2000, there have been several case-control studies assessing self-report of sun exposure throughout the life course [46–53]. Though these studies vary in their populations, sample sizes, assessments of past or lifetime sun exposure, and statistical methods, they collectively support that higher sun exposure, in particular during childhood and adolescence, is associated with a reduced risk of MS. While recall bias of self-reported sun exposure behaviors and time in the past is a concern, the studies using actinic damage to the dorsum of the hand—an objective measure of cumulative sun exposure—also find a lower MS risk.

In a study of Australian MS cases and controls [46] increasing actinic damage was inversely associated with MS risk (grade 6 vs. grade 3 OR = 0.17; 95% CI(0.05–0.60)). These findings were confirmed in the larger Ausimmune study (grade 6 vs. grade 2 OR = 0.43; 95%CI(0.21–0.88)) among individuals who had experienced their first demyelinating event [53]. Whether the inverse associations seen in these studies are driven by vitamin D pathways, other sun-induced immune-associated pathways, or both, has been debated [54,55]. Two studies reported that sun exposure and 25(OH)D levels are independent risk factors for MS, suggesting a role for sun exposure in reducing MS risk via non-vitamin D-related mechanisms [53,56]. However, in these studies, vitamin D levels were measured in blood samples taken at the time of the study and not

during the reported time period of sun exposure (in childhood/adolescence) so both associations may still be explained by vitamin D. PhoCIS was an Australian randomized controlled clinical trial of the effects of UVB phototherapy on conversion to MS among patients with a CIS [57]. All participants were also supplemented with vitamin D to raise levels higher than 80 nmol/L. After 12 months, the study found no statistically significant difference in the rate of conversion with phototherapy versus none. One possible explanation is that vitamin D levels were set high via supplementation and further exposure to UVR had no great effect, suggesting that the vitamin D pathway via sun exposure is probably the main mechanism of the effects of sun exposure on MS risk.

### 4.2 Dietary intake of vitamin D and MS risk

Numerous case-control studies have reported that individuals with a high intake of foods or supplements that are rich in vitamin D have a lower risk of MS. Studies in Norway [37], Sweden [58,59], and Australia [60] found a 20%–40% decreased risk of MS among regular consumers of fish and a study on cod liver oil intake during adolescence reported a 30% reduced risk [61]. However, the case-control study design is not optimal for assessing diet-disease associations as they are particularly prone to selection and recall biases [62]. Further, foods that are rich in vitamin D tend to also be rich in other nutrients which may have a role in MS development including polyunsaturated fatty acids [63,64] and vitamin A [65].

Longitudinal prospective studies of diet and disease are much less likely to be biased by selection or recall and they are the preferred observational study design for assessing associations between diet and disease [66]. In the Nurses' Health Study (NHS) and NHS II, two longitudinal cohorts of over 200,000 women, dietary intake are assessed every 4 years using extensively validated food frequency questionnaires [67]. The dietary foods and nutrients used in a prospective analysis are those collected before the woman develops the disease of interest. In the NHS and NHSII, pre-diagnostic dietary intake of >400 IU/day of vitamin D as multivitamin supplements was associated with a 40% decreased risk of MS when compared to women who did not use multivitamin supplements [68]. However, as with the case-control studies, other nutrients in the multivitamins may be confounding the association. Another important consideration is that the contribution of dietary vitamin D (including supplements) to overall vitamin D status is less than vitamin D derived from sun exposure. While the studies of dietary vitamin D via food and supplements support the hypothesis, they provide weak evidence of a potential protective effect of adequate vitamin D nutrition on MS risk.



### 4.3 25-Hydroxyvitamin D (25[OH]D) and MS risk

The limitations of dietary vitamin D intake and sun exposure as proxies for complete vitamin D nutrition allow only for indirect evidence that vitamin D specifically is driving the decreases in MS risk observed. Circulating blood levels of 25-hydroxyvitamin D (25(OH)D) are an integrated measure of vitamin D from both diet and sun exposure and provide a more direct assessment of vitamin D nutrition. Studies have found low 25(OH)D levels in persons with MS [69]. However, reverse causation is a plausible explanation of the results as blood samples are taken after the onset or diagnosis of MS or CIS (CIS—very early MS) has been made and the MS disease process or behavior changes in persons with MS, such as sun avoidance, may be the reason for the lower vitamin D levels observed.

To address this limitation, prospective nested case-control studies measuring circulating levels of 25(OH)D in healthy individuals and following them for an MS diagnosis have been conducted in four well-defined populations, and all are consistent with sufficient 25(OH)D levels associated with a decreased risk of MS [70–73] (Fig. 102.1). A study among active-duty US military personnel [70] documented 257 MS cases and matched them to 514 controls on age, sex, race, dates of sample collection, and branch of military service. For each case and control, up to three serum samples collected before the date of MS symptom onset (index date in controls) were obtained from the Department

of Defense Serum Repository, which stores over 65 million serum samples from over 10 million personnel collected periodically over the course of their military careers. There was a median time of 4 years between sample collection and MS onset. Among NHW personnel, pre-symptom 25(OH)D levels  $>100$  nmol/L was associated with a 50% decreased risk of MS. This association was not seen among NHB or Hispanics, but this may be due to smaller sample sizes and a limited range of 25(OH)D levels in these groups.

There have been two studies from Sweden utilizing various biobanks in the country. The first used serum samples stored by the North Sweden Maternity Cohort and the North Sweden Health and Disease Study [71]; 192 MS cases and 384 controls matched on sex, biobank, sampling date, and age had a serum sample stored that was collected prior to the date of MS onset (index date in controls) with the samples collected a median of 9 years before the onset of MS. Levels of 25(OH)D  $>75$  nmol/L were associated with a 60% reduced MS risk. The second study was conducted using five Swedish university hospital biobanks and one public health biobank; [73] 665 MS cases and 655 controls matched on biobank, sex, date of sample collection, and age had samples collected a median of 8 years before onset. Sufficient 25(OH)D levels ( $\sim >70$  nmol/L) were associated with a 32% decreased MS risk.

The studies in the United States and Sweden all support that adequate vitamin D nutrition in otherwise healthy young adults is associated with a decreased risk of MS. A study in the Finnish Maternity Cohort

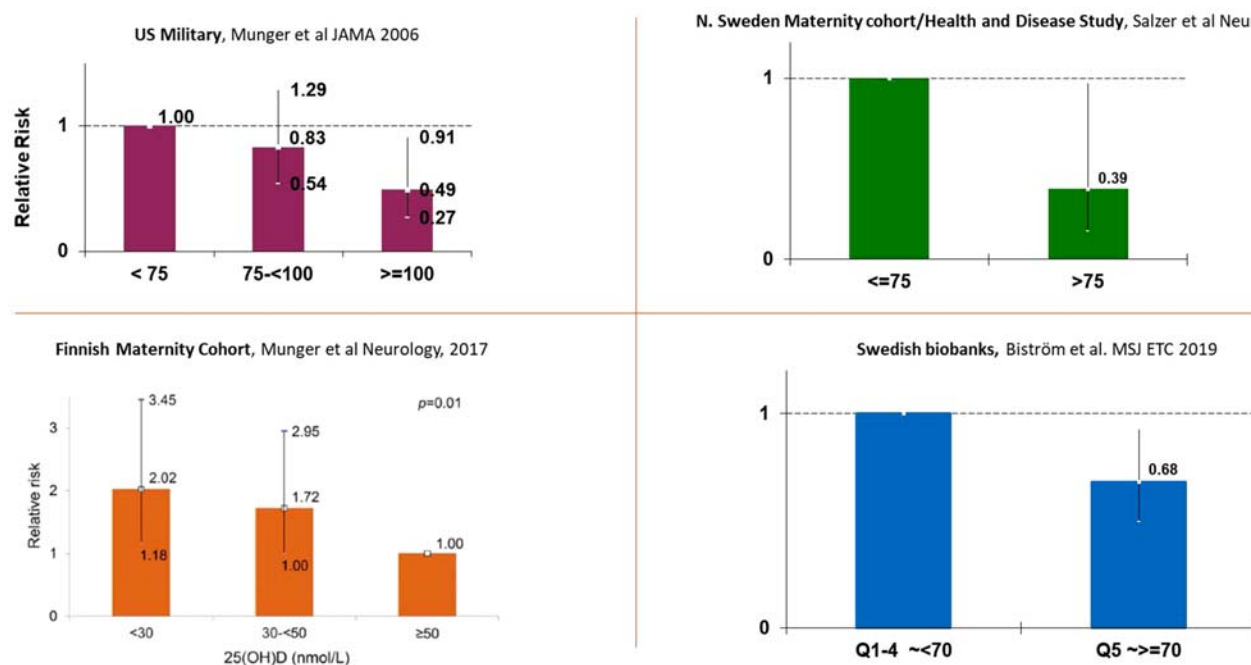


FIGURE 102.1 Main results from the prospective studies of 25(OH)D and MS risk.

(FMC) examined whether vitamin D insufficiency ( $<50$  nmol/L) or deficiency ( $<30$  nmol/L) was associated with an increased risk of MS [72]. In the previous studies, too few individuals had low enough 25(OH)D levels to study this end of the range. The FMC has collected and stored serum samples obtained during the first trimester of  $>98\%$  of all pregnancies in Finland since 1983 [74]. Linking the FMC to hospital and prescription registries, 1092 women with MS had at least one sample stored in the FMC that was collected prior to the symptom onset of MS. They were matched to 2123 controls on birthdate and area of residence, and the mean time between sample collection and MS onset was 9 years. Over 50% of the women had 25(OH)D levels in the deficient range and both deficient and insufficient levels of 25(OH)D were associated with a twofold increased risk of MS.

As mentioned above, these studies are not subject to recall bias and selection bias is minimized by the nested case-control study design. However, confounding and reverse causation as explanations for the findings need to be considered. The time lag between pathological onset and MS symptom onset is referred to as the “prodromal phase” of MS [75]. Various studies have estimated this phase may begin 5–10 years before classic MS symptoms appear, implying that in the prospective studies, there are likely some 25(OH)D levels that are being measured within this time frame and the 25(OH)D levels may be affected by the subclinical disease process. Studies of early life 25(OH)D exposure and future risk of MS may help to clarify given the long lag time between early life exposure and adult-onset MS.

Ueda et al. [76] conducted a case-control study in Sweden of 459 MS cases and 663 controls who had neonatal dried blood spots (DBS) stored since their birth in 1975 or later in the Swedish Phenylketonuria Register. They measured 25(OH)D levels in the DBS and found no association between neonatal 25(OH)D levels and future risk of MS. Several limitations of the study, such as degradation of some DBS sampled and low control participation leading to a distortion of the true 25(OH)D exposure distribution of the source population, may have contributed to a lack of association in this study [77].

There have been two prospective nested case-control studies on early life exposure to 25(OH)D and the risk of MS in adulthood. One was conducted in the FMC using samples collected during the pregnancy with the offspring who later developed MS [77] and the other in the Danish MS registry among individuals with stored neonatal dried blood spots [78]. Both studies found a 1.5- to twofold increased risk of adult-onset MS among offspring with evidence of exposure to 25(OH)D levels  $<30$  nmol/L during gestation. These studies suggest that vitamin D nutrition in early life is

also important for the risk of future MS and that the observed associations between 25(OH)D in young adulthood and MS in prospective studies may not be entirely explained by reverse causation.

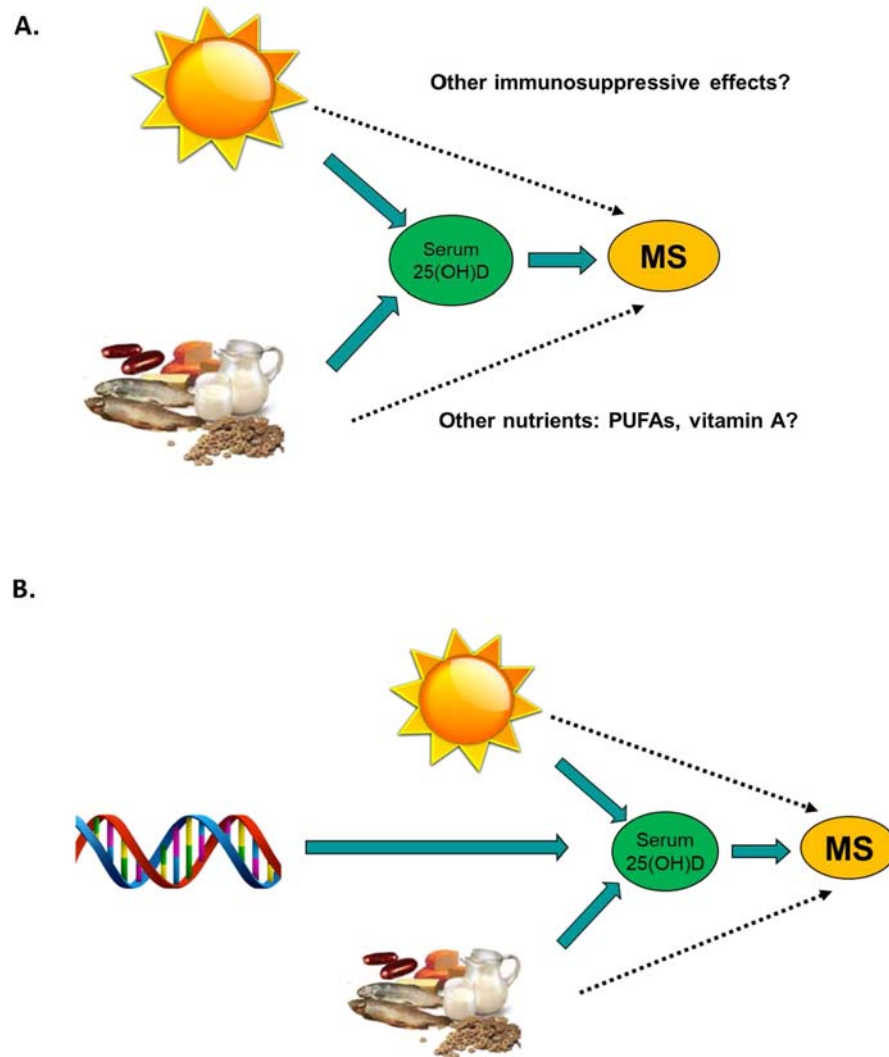
Confounding of the 25(OH)D—MS association by sun exposure via non-vitamin D pathways or other nutrients in vitamin D-rich foods, such as polyunsaturated fatty acids, is plausible (Fig. 102.2). Mendelian randomization (MR) studies minimize confounding as an explanation of observed associations by taking advantage of the fact that genes are randomly distributed before any confounding exposures occur. The sorting of gene variants that contribute to determining 25(OH)D levels are therefore not associated with sun exposure or diet and cannot confound the 25(OH)D—MS risk association. Six MR studies have been conducted and all have found an association between higher genetically determined 25(OH)D levels and lower MS risk [79–84] suggesting that confounding cannot completely explain the 25(OH)D and MS risk association in the prospective nested case-control studies. Importantly, however, MR studies also do not prove there is a causal association and they are limited by untestable or unprovable assumptions [85]. However, multiple studies in different populations with similar results suggest these assumptions are met or have minimal impact. More details of these studies are provided below and in Table 102.1.

#### 4.4 Vitamin D genes and MS risk

Several genes related to vitamin D metabolism and action have been assessed with respect to MS risk including *DHCR-7*, *VDBP*, *VDR*, *CYP27B1*, and *CYP24A1*. Results of these candidate gene studies have been mixed and there are no unequivocal associations between polymorphisms in these genes and MS risk. MR studies, however, create instrumental variables using gene variant predictors of 25(OH)D levels from large genome-wide association study (GWAS) and assess whether there are associations between genetically determined levels of 25(OH)D and MS risk. These results have been consistent in showing that genetically determined higher levels of 25(OH)D are associated with lower MS risk. A more detailed description of MR and studies of vitamin D and human health is provided in Chapter 61, but a summary of the key genes linked to vitamin D and MS is shown below.

##### 4.4.1 *NADSYN1/DHCR-7*

The *NADSYN/DHCR-7* locus on chromosome 11 has been associated with vitamin D levels in GWAS [86,87]. The *DHCR-7* gene encodes the enzyme 7-dehydrocholesterol reductase which converts 7-dehydrocholesterol to cholesterol. 7-dehydrocholesterol is also the precursor



**FIGURE 102.2** (A) Schematic of potential confounding of the association between 25(OH)D and MS risk by other immunosuppressive actions of UVR exposure and other nutrients found in foods with vitamin D. (B) Schematic of the Mendelian randomization studies. Using genes that predict 25(OH)D levels as instrument variables eliminate confounding by other properties of UVR or dietary intake of vitamin D.

to vitamin D produced via UVB exposure. High levels of 7-dehydrocholesterol reductase may result in low vitamin D levels as 7-dehydrocholesterol is preferentially converted to cholesterol. Results of the few studies conducted suggest that there is no association between SNPs in this locus and MS risk [88–90].

#### 4.4.2 *CYP2R1*

The *CYP2R1* gene, also located on chromosome 11, encodes the 25-hydroxylase enzyme which converts vitamin D to 25(OH)D in the liver. Two studies have found an increased MS risk associated with SNP rs10766197 in Italian [89] and Spanish [91] populations. The rs117913124 SNP was associated with an increased MS risk in a White European population [92]. No associations between other SNPs in *CYP2R1* and MS risk have been observed [88,89,93].

#### 4.4.3 *CYP27B1*

The *CYP27B1* gene encodes the  $1\alpha$ -hydroxylase enzyme that converts 25(OH)D to the biologically active 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ). A 2009 GWAS by the Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene) first identified three SNPs on chromosome 12q13-14 associated with MS risk: rs703842, rs10876994, and rs1236853 [94]. In a 2011 GWAS, the International Multiple Sclerosis Genetics Consortium (IMSGC) also reported an association with rs1236853, but not rs703842 or rs10876994 [24]. A non-GWAS study among Han Chinese also found an association between rs1236853 and MS risk [95]. Other studies including the rare *CYP27B1* variants rs118204009, rs118204011, rs118204012, rs12368653, and rs10876994 have produced mixed results and no clear consistent associations with MS risk [91,96–101]. In

**TABLE 102.1** Overview of Mendelian randomization studies of genetically determined 25(OH)D levels and risk of MS.

Study	25(OH)D GWAS (n, population)	MS population	25(OH)D gene-related SNPs included	MR OR (95% CI), <i>P</i> value
Mokry et al. [79]	SUNLIGHT (n = 33,996, European)	IMSGC (n = 14,498 MS/24,091 controls)	rs2282679, rs12785878, rs10741657, rs6013897	2.0 (1.7–2.5) $P = 7.7 \times 10^{-1}$ for a 1 SD decrease in 25(OH)D levels
Rhead et al. [80]	Genetic risk score from three previously published SNPs	KPNC (US) and EIMS/GEMS (Sweden) (Total n = 7391 MS/14,777 controls)	rs2282679, rs2060793, rs3829251	0.85 (0.76–0.98) $P = .003$ for each increase in number of alleles associated with higher 25(OH)D levels.
Gianfrancesco et al. [81]	Genetic risk score from three previously published SNPs	US (n = 394 pediatric-onset MS/10,875 controls) and Sweden (175 pediatric-onset MS cases and 5736 controls).	rs2282679, rs2060793, rs3829251	0.72 (0.55–0.94) $P = .02$ for each increase in the number of alleles associated with higher 25(OH)D levels.
Jacobs et al. [82]	SUNLIGHT (n = 79,366, European)	IMSGC (n = 14,802 MS, 26,703 controls)	rs8018720, rs3755967, rs79798805, rs12785878, rs107416557, rs17216707	0.57 (0.41–0.81), $P = .001$ for a 1 SD decrease in 25(OH)D levels
Harroud et al. [84]	UK biobank (n = 401,460 British)	IMSGC (n = 14,802 MS, 26,703 controls)	88 SNPs	0.72 (0.60–0.87) $P = 6.2 \times 10^{-4}$ for a 1 SD increase in 25(OH)D
Wang [83]	UK biobank (n = 401,460 British)	IMSGC (n = 14,498 MS, 24,091 controls)	20 SNPs	

*CI*, confidence interval; *EIMS/GEMS*, Epidemiologic Investigation of Multiple Sclerosis/Genes and Environment in Multiple Sclerosis; *GWAS*, Genome-wide association study; *IMSGC*, International Multiple Sclerosis Genetics Consortium; *KPNC*, Kaiser Permanente Northern California; *OR*, odds ratio; *SD*, standard deviation; *SNPs*, single nucleotide polymorphism.



contrast, studies of the rs703842 SNP (C vs. T) in the 3' UTR have shown a consistent inverse association with MS risk [93,95,96,102–106], and a meta-analysis of the pre-2019 studies reported an odds ratio = 0.85, 95%CI: 0.80–0.89 for MS risk associated with carrying the “C” allele [107].

#### 4.4.4 DBP

As a major determinate of vitamin D levels, variants of the vitamin D binding protein (DBP, also known as Group-specific component or Gc) have been studied with respect to MS risk. Two SNPs in exon 11, rs7041 and rs4588, lead to variation in the DBP such that there are three main DBP haplotypes: Gc1f, Gc1s, and Gc2. Gc1f is associated with higher 25(OH)D levels and is more common in Blacks than Whites, Gc1s is associated with lower 25(OH)D levels and occurs more frequently in Whites than Blacks [108]. Two studies of the Gc alleles in 1988 found no association between them and MS risk [109,110]. Subsequent studies also failed to find any associations between rs7041 and rs4588 and the risk of MS [99,103,104,111,112].

#### 4.4.5 CYP24A1

The *CYP24A1* gene encodes the 24-hydroxylase, the enzyme that deactivates 25(OH)D and 1,25(OH)<sub>2</sub>D by adding a hydroxyl group to carbon 24. In the ANZgene GWAS [94], SNPs on chromosome 20q13 were associated with MS risk. Though there are other candidate genes in the region (e.g., CD40), *CYP24A1* also maps to this location. In the IMSCG GWAS [24], rs2248359 on chromosome 20 was significantly associated with MS risk and mapped to *CYP24A1*, suggesting that the identification of 20q13 as a locus of interest in the ANZgene study may have been picking up an association with *CYP24A1*. The rs2248359 SNP is in the promoter region of *CYP24A1* and regulates the expression of the gene. However, a candidate gene study conducted in a Han Chinese population found no association between this promoter SNP and MS risk [95]. A study of the rs2296241 SNP found no association with MS risk [93], but an Italian study reported an increased risk of MS with homozygosity of the minor allele “C” in rs22481837 [113], which is associated with low 25(OH)D levels. A transmission disequilibrium analysis of 16 *CYP24A1* SNPs found no evidence of MS risk associated with these SNPs in a Canadian population of MS cases and first-degree relatives [103].

#### 4.4.6 VDR

The vitamin D receptor gene, *VDR*, is of particular interest with respect to MS risk as it is a nuclear receptor that binds vitamin D response elements in DNA thereby regulating the expression of genes in a variety of cell types, including those of the immune system. There

are four restriction fragment length polymorphisms: *ApaI* (rs7975232), *BsmI* (rs1544410), *FokI* (rs2228570), and *TaqI* (rs731236). While the *VDR* is the most studied vitamin D-related gene with MS risk, studies have generated mixed results on the associations between these RFLP and MS risk [93,114–140]. These differences are likely due to combinations of small sample sizes in the case-control studies, low power, and the race, ethnicity, or location of the population included. There have been several meta-analyses of the studies on *VDR* RFLPs and MS risk published since 2011 [123,140–143]. In two from 2019, one included 27 studies [142] and one 30 studies [143]. For the RFLP *ApaI*, both meta-analyses reported no statistically significant associations with MS risk in White populations, but in Asians, a reduced MS risk with having *ApaI* was seen. In Zhang et al.'s analysis, there was no association between the RFLP *BsmI* either overall or in population subgroups, whereas Imani et al. reported no association in Whites, Asians had a statistically significant 78% increase in MS risk if they carried two *BsmI* variants. The reasons for this difference are not clear but may be due to one meta-analysis having included three studies the other did not, as well as differing interpretations or errors of the original data. The *TaqI* RFLP was significantly associated with a lower MS risk in White populations and no associations were found in Asians. The *FokI* RFLP was inversely associated with MS in both White and Asian populations in one meta-analysis [142], but not the other [143].

#### 4.4.7 Mendelian randomization (MR) studies

As discussed above, the MR studies of 25(OH)D and MS risk lend support for the causality of the observational study findings that high levels of 25(OH)D are associated with a reduced risk of MS as they minimize both confounding and reverse causation (see Chapter 61). Additionally, the MR studies support the role of genetic variants in vitamin D metabolism-associated genes. Where the results of candidate gene case-control studies described above have been largely equivocal, the inverse associations between genetically determined 25(OH)D levels and MS risk reported in the 6 MR studies conducted to date provide strong evidence that vitamin D-related genes impact MS risk.

The salient features of the 6 MR studies that have been conducted are shown in Table 102.1. Four [79,82–84] of the six studies used large 25(OH)D GWAS—SUNLIGHT and UK Biobank—for discovery and the IMSCG to test the association between the 25(OH)D level associated SNPs and MS risk. Two studies utilizing data from the US and Sweden [80,81] created a genetic risk score based on three SNPs known to affect 25(OH)D levels and reported a decreased risk of MS with an increasing number of alleles associated with

higher 25(OH)D levels in both adult- [80] and pediatric-onset [81] MS. Collectively, the MR studies strongly support a role for vitamin D in MS development. However, all of these studies were conducted in largely White, European populations, so whether these results extend to other races and ethnicities requires study. Further, they do not inform at what stage of life higher 25(OH)D levels may be important for determining MS risk or whether 25(OH)D levels affect MS outcomes.

## 5. Vitamin D, race, and MS risk

The vast majority of all the research described above has been conducted in largely White populations of European descent. In the prospective nested case-control study of 25(OH)D and MS risk in the US military the distribution of 25(OH)D was too narrow and the sample size among Blacks with MS too small to evaluate the risk of MS with vitamin D in this group. As described elsewhere in detail, endogenous production of vitamin D with exposure to UVB is inversely proportional to the amount of melanin pigmentation in the skin; therefore, Blacks, especially those living in more northern latitudes, have a high risk of vitamin D deficiency [144]. Studies among Kaiser Permanente members in Southern California [56] reported that levels of 25(OH)D in one sample collected after MS onset were not associated with MS risk in Blacks, but they were inversely associated with MS in Whites. Further, a lifetime history of sun exposure was associated with a reduced risk of MS in both Blacks and Whites. The authors concluded these results were more consistent with non-vitamin D pathways of UVB exposure being more relevant to MS risk than vitamin D itself. However, an assessment of lifetime exposure to the sun is a better measure of overall vitamin D status than one measure taken in adulthood and the results are also consistent with an inverse association of vitamin D with MS risk in Blacks. Much more research on vitamin D as a risk factor for MS in non-White populations is needed [144].

## 6. Vitamin D and MS disease activity and progression

Despite effective therapies that reduce relapse rates and the development of new lesions, the progression of relapsing-remitting MS to secondary progressive MS occurs in most cases. As such, whether adequate vitamin D nutrition may favorably modify MS activity or progression is an active area of research. MS “activity” refers to more acute manifestations of the illness such as conversion from a CIS to definite MS, rate of relapse

**TABLE 102.2** Expanded disability status scale (EDSS) for multiple sclerosis (see Ref. [145] for more detail).

- 
- 0 = Normal
  - 1–1.5 = No disability, but some abnormal neurological signs
  - 2–2.5 = minimal disability
  - 3–4.5 = Moderate disability, affecting daily activities, but you can still walk
  - 5–8 = more severe disability, impairing your daily activities and requiring assistance with walking
  - 8.5–9.5 = very severe disability, restricting you to bed
  - 10 = Death
- 

occurrence, and the appearance of new demyelinating lesions in the CNS visible on magnetic resonance imaging (MRI). MS progression is a more chronic phase of the disease and is caused by the accumulation of neurological deficit that is typically measured by the sustained increase (usually at least 6 months) of disability on the Expanded Disability Status Scale (EDSS) [145] (Table 102.2).

Other measures such as brain atrophy and cognitive function also provide insight into MS progression. There have been both observational studies and randomized clinical controlled trials on vitamin D and MS outcomes. Common criticisms of the observational studies include confounding and reverse causation biases and as a result cannot show that vitamin D has a direct effect on clinical or MRI outcomes. The RCTs also pose challenges to interpretation primarily given their heterogeneity in the vitamin D intervention employed and disease-modifying therapies the participants use, sample size (and thus power), and follow-up time to observe MS outcomes which vary from as little as 6 up to 22 months (Table 102.3). Interpretation and comparison of the results from both study designs must be within the context of these main limitations.

### 6.1 Conversion from CIS to MS

#### 6.1.1 Observational studies

Several studies have shown that insufficient or deficient 25(OH)D levels are associated with a higher rate of conversion from a CIS to clinically definite MS (CDMS) [156–158]. In an Italian study [156], 100 patients with CIS were followed for a median of 7 years and levels of 25(OH)D 32.7 nmol/L were associated with a 2–3fold increased risk of conversion to CDMS as compared to CIS patients with 25(OH)D levels between >32.7 and <71.6 nmol/L. Similarly, a European study of 1047 CIS patients followed for a median of 4 years found that those who were in the bottom quartile of 25(OH)D

**TABLE 102.3** Randomized controlled clinical trials of vitamin D and MS outcomes.

Author, year	n	Duration	Vitamin D intervention	25(OH)D level (nmol/L) at end of study treated versus placebo <sup>a</sup>	Main results
Burton (2010) [146], Kimball (2011) [147]	49	12 months	Cholecalciferol increased from 4000 IU/day to 40,000 IU/day over 28 weeks, followed by a reduction to 10,000 IU/day for 12 weeks and to 4000 IU/day for 6 weeks Calcium: 1200 mg/day; placebo: Up to 4000 IU vitamin D/day and calcium	200 versus not reported	In treated versus non-treated, nonsignificant reduction in annualized relapse rate (0.26 vs. 0.45) and proportion of patients experiencing a relapse (0.16 vs. 0.37); suggestion of lower EDSS at EOS for vitamin D group (1.15 vs. 1.45)
Soilu-Hänninen (2012) [148]	66	12 months	Cholecalciferol 20,000 IU/wk all using IFNB-1b	110 versus 50	Nonsignificant reduction MRI T2 burden of disease ( $P = .1$ ); number of T1 Gd enhancing lesions significantly decreased ( $P = .004$ ). Nonsig. Favorable effects on EDSS ( $P = .07$ ) and timed tandem walk ( $P = .07$ ).
Kampman (2012) [149]	68	22 months	Cholecalciferol 20,000 IU/wk. Treated and placebo groups were given 500 mg calcium and all allowed to continue routine vitamin D supp.	123 versus 62	No difference in relapse rate, EDSS, or MSFC changes
Stein (2011) [150]	23	6 months	Ergocalciferol 6000 IU/d placebo: 1000 IU/d	6000 IU/d: 120 (median) 1000 IU/d: 69	Increase in EDSS and relapses in high-dose vit D group
Mosayebi (2011) [151]	62	6 months	Cholecalciferol 300,000 IU/month all using IFNB-1a	~140 versus ~25	No difference in EDSS or number of Gd-enhancing lesions
O'Connell (2017) [152]	29 CIS	24 weeks	Cholecalciferol 5000 IU/d or 10,000 IU/d	5000 IU/d: 129 10,000 IU/d: 168 Placebo: 71	No difference in any clinical or imaging outcomes
Hupperts (2019) [153]	229	48 weeks	Cholecalciferol 6670 IU/d 1st 4 wks, 14,007 IU/d 44 wks. All participants using IFNB-1a	216 versus 50 (median)	No difference in NEDA-3 at wk 48 (36.5% vs. 35.3%). Lower rate of combined unique activity lesions (IRR = 0.68, $P = .005$ ) and decrease in T2 lesions volume ( $P = .04$ ) in vitamin D group
Camu (2019) [154]	129	96 weeks	Cholecalciferol 100,000 IU every other week. All using IFNB-1a	157 versus 57	ITT: no association with annualized relapse rates. In completers: Decreases were observed for relapse rate, new T1 lesions, and T1 lesion volume
Dörr (2020) [155]	53	18 months	Cholecalciferol every other day 20,400 versus 400 IU. All using IFNB-1b	20,400 IU: 162 400 IU: 56	No associations with any clinical or imaging outcomes

<sup>a</sup>mean level unless otherwise noted.

had a faster rate of conversion to CDMS as compared to those with higher vitamin D levels [157]. A study within the BENEFIT trial of early versus late administration

interferon- $\beta$ 1b among 465 CIS patients found that those with an average of 25(OH)D levels <50 nmol/L over the first year of the trial had a higher probability of CDMS

over years 2-5 of the trial [158]. CDMS is diagnosed in CIS patients after they experience a second clinical attack (relapse) of neurologic deficit lasting for at least 24 h in the absence of fever or infection [5]. The inverse associations between 25(OH)D levels and progression from CIS to CDMS suggest that 25(OH)D may also have a beneficial effect on relapse rate among persons with MS.

### 6.1.2 Randomized control trials (RCT)

There has only been one trial of vitamin D in persons with a CIS [152]. Participants were randomized to either receive cholecalciferol 5000 IU/d, 10,000 IU/d, or placebo. Over the 24 weeks of the study, there was no difference on clinical or imaging outcomes between these groups. However, the short duration and small sample size ( $n = 29$ ) may in part explain the null results.

## 6.2 Relapse rates

### 6.2.1 Observational studies

In general, persons with MS tend to avoid prolonged sun exposure as the heat can exacerbate their symptoms, which in turn can lead to vitamin D deficiency or insufficiency in MS patients. Several studies have found that low blood 25(OH)D levels are predictive of an increase in MS relapse rate [159–164]. One important caveat of these studies, however, is that reverse causation may explain the results—that is, persons with MS and high vitamin D levels may have less active disease and are able to tolerate sun exposure or actively take a vitamin D supplement. A study among individuals with a CIS in the BENEFIT trial minimized this limitation by using average 25(OH)D levels in the first year of the trial as a predictor of relapse rate in years two through five; average 25(OH)D levels  $\geq 50$  nmol/L were associated with a 57% decrease in risk of annualized relapses during the follow-up [158]. However, no association was seen between 25(OH)D levels and annualized relapse rate among RRMS participants in the BEYOND trial [165]. Possible explanations for the seemingly conflicting result include that participants in BEYOND had established MS with a median time since onset of 3 years (range  $<1$  to  $>10$  years) at baseline and that the follow-up was only to year two of the trial as compared to year five in BENEFIT.

### 6.2.2 RCTs

Overall, RCTs have not found an association between vitamin D supplementation and reduced annualized relapse rates (Table 102.3). Some explanations for the inconsistency with the observational studies include a shorter duration of follow-up and low statistical power to detect an association.

## 6.3 Expanded Disability Status Scale (EDSS)

### 6.3.1 Observational studies

The Expanded Disability Status Scale (EDSS) is a commonly used measure of disability progression in MS studies. A brief summary of the EDSS is shown in Table 102.1, and Kurtzke [145] provides a detailed and in-depth definition of each step (0 and half-steps from 1–10). A patient's EDSS is assessed by a neurologist or MS specialist, and the lower the score, the lower the disability experienced by the patient at that point in time. Most of the cross-sectional studies that measure 25(OH)D levels at the same time as the EDSS have found an inverse association between them [164,166–169]. However, whether this inverse association reflects 25(OH)D as a risk factor for EDSS progression or if the greater disability leads to less time outdoors or in the sun resulting in lower 25(OH)D levels cannot be determined from these studies. In prospective studies where 25(OH)D is measured as a predictor of future EDSS results has been mixed. In the BENEFIT study [158], higher average 25(OH)D levels in year 1 were associated with less progression on the EDSS in years 2–5. In the BEYOND study [165], there was a non-statistically significant lower risk of EDSS progression among those with higher 25(OH)D levels over a median of 2 years of follow-up.

### 6.3.2 RCTs

No associations between vitamin D supplementation and EDSS were reported, with the exception of the RCT by Stein et al. [150] (Table 102.3). However, several limitations to this trial, including imbalances between the treated (high dose) and nontreated (low dose) groups in age and sex were not accounted for in the analyses.

## 6.4 MRI

### 6.4.1 Observational studies

Favorable MRI outcomes, including fewer number of new or active CNS lesions and lower lesion volume among CIS or RRMS patients with higher levels of 25(OH)D have been observed [158,163,165,170] (Table 102.4). While the magnitudes of the relative risks are for a range of 25(OH)D increments across studies, there is convergence in that as 25(OH)D levels increase, the risk of developing new T2 Lesions, new gadolinium-enhancing (active) lesions and a cumulative number of active lesions among MS patients decreases. Studies have also assessed whether 25(OH)D levels predict T2 lesion volume or brain volume loss. Ascherio et al. [158] reported that participants in the BENEFIT trial with an average 25(OH)D in the first year  $\geq 50$  nmol/L had a 9% reduction in T2 lesions volume between years



**TABLE 102.4** Summary of 25(OH)D levels and MRI outcomes in CIS/RRMS patients—prospective studies.

Author, year	n	Duration	25 (OH) D unit	New T2 lesions	New active (Gd +) lesions	Cumulative # new active lesions
Mowry (2012) [163]	469	1 year	25 nmol/L increase	RR = 0.85 (0.76–0.95), P = .004	RR = 0.68 (0.53–0.87), P = .002	NA
LøkenAmsrud (2012) [170]	88	6 months	10 nmol/L increase	RR = 0.88 (0.78–1.0), P = .04	RR = 0.87 (0.77–0.99), P = .04	NA
Ascherio (2014) [158]	464	48 months	> = 50 nmol/L versus <50 nmol/L	NA	RR = 0.43 (0.26–0.70), P = <0.001 per 50 nmol/L increase	RR = 0.73 (0.60–0.89), P = .002
Fitzgerald (2015) [165]	1482	24 months	50 nmol/L increase	NA	NA	RR = 0.69 (0.55–0.86), P = .001

two and five and a 0.34% lower yearly loss in brain volume over the same time, as compared to those participants with 25(OH)D levels <50 nmol/L. In the BEYOND trial, no association between 25(OH)D levels and brain volume was observed but the follow-up of 2 years may have been too short to see any meaningful changes [165].

Several of the RCTs found evidence in support of vitamin D supplementation and beneficial effects on MRI outcomes including reduction in CNS lesion burden and lesion volume (Table 102.3), though most were underpowered to detect statistically significant differences between the groups. While two recent trials, SOLAR [153] and CHOLINE [154], did not meet their primary outcomes, both suggested that lower CNS lesion burden was achieved in the high-dose vitamin D groups as compared to placebo (Table 102.3).

Collectively, the strongest evidence from the observational and RCT studies is that vitamin D has beneficial effects on CNS lesion development and volume both early in the disease process as well as in RRMS patients with established disease. The demyelinated areas of the CNS are indicative of an underlying immune and inflammatory process that drives the destruction of the myelin sheaths and axons of the nerve cells. The convergence of the prospective observational and RCT findings on MRI outcomes suggests that vitamin D likely has a role in affecting this immune and inflammatory response.

## 7. Pediatric onset MS

An estimated 3%–10% of all MS cases experienced symptoms prior to age 18 and have pediatric-onset MS [171]. As with adult-onset MS, pediatric-onset MS is also associated with the HLA-DRB1\*1501 risk allele [172], infection with EBV [172], and being obese/overweight in childhood [173,174]. Vitamin D as a risk factor and modifier of disease activity has also been studied in

this population, though not as extensively as in the adult-onset population. Reasons for this may include the smaller population or no pre-onset blood samples available to conduct prospective studies.

Abundant sun exposure in childhood and adolescence has been associated with a lower risk of adult-onset MS as described above. However, there have been no studies looking at the link between sun exposure and pediatric MS onset. A study in Canada [172] included 302 children with an incident demyelinating event for a median of 3 years. Blood levels of 25(OH)D were measured in samples collected within 40 days of the onsetting event. Over the course of the follow-up, 21% of the children were diagnosed with MS and risk was increased among those with lower 25(OH)D levels (per 10 nmol/L decrease in 25(OH)D: HR = 1.11, 95% CI: 1.00–1.25). A MR study of vitamin D and pediatric MS risk found that genetically determined higher 25(OH)D levels were associated with a 28% decreased risk [81].

Three studies have suggested that higher 25(OH)D levels are associated with a lower relapse rate in pediatric MS. Mowry et al. [175] reported that in 110 children with MS or CIS followed for a median of 1.7 years from blood collection that every 10 ng/mL increase in 25(OH)D was associated with a 34% reduced relapse rate. A study in 181 pediatric MS and CIS cases suggested that the association between vitamin D and relapse rate may be modified by HLA-DRB1 alleles as a reduced relapse rate with increasing 25(OH)D was only seen among carriers of HLA-DRB1\*1501 or \*1503. There is also evidence suggesting that having genetically lower 25(OH)D is also associated with a twofold increase in relapse rate in pediatric MS [176].

The current evidence does support a role for vitamin D nutrition in both risk of pediatric-onset MS as well as in relapse occurrence among children with MS. Further studies confirming these findings as well as researching how race/ethnicity, sex, or other MS risk factors may

modify these associations are warranted. Observational or RCT studies on vitamin D supplementation and other MS outcomes also need to be considered.

## 8. Conclusion

There is strong evidence supporting vitamin D nutrition as a risk factor for MS, as well as vitamin D having beneficial effects on MS disease activity and progression, but many questions remain. Associations between the level of prenatal vitamin D exposure, sun exposure in childhood and adolescence, and 25(OH)D levels in young adulthood suggest that vitamin D levels in early life are important in determining MS risk, but is one life stage a more critical period than another? The feasibility of conducting an RCT of vitamin D and primary prevention of MS has been debated. Some of the challenges to design include whether an RCT should be in the general population or only among first-degree relatives of persons with MS, which begs the question of whether it is ethical to withhold high vitamin D doses from first-degree relatives given the observational data. What doses of vitamin D should be tested? Given MS is a rare disease, even among first-degree relatives, an RCT would require a large sample size and a long duration of follow-up, especially if early-life exposure is thought to be critical, which may affect treatment compliance and significantly increase costs. The majority of MS cases and controls in these studies are White and of European descent. There needs to be more research into whether vitamin D is also a risk factor for MS in other race/ethnic populations including Blacks and Hispanics. The evidence from observational studies and RCTs on the effects of vitamin D on MS disease activity and progression suggests that having high 25(OH)D levels reduces the development of new demyelinating lesions. Results on other outcomes have been mixed and well-designed RCTs of appropriate follow-up and power are needed to evaluate this, but significant challenges, including recruitment, costs, and appropriate vitamin D supplement dose to use, remain barriers.

## 9. Summary points

- There is strong evidence that vitamin D nutrition is a risk factor for MS as well as disease activity.
- 25(OH)D levels are inversely associated with both adult- and pediatric-onset MS.
- Sun exposure in childhood and adolescence is inversely associated with adult-onset MS.
- Among individuals with MS, evidence suggests that vitamin D beneficially affects relapse rates and new lesion development.

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# Vitamin D and rheumatoid arthritis

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## OBJECTIVES

- Describe the immune interactions of 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D that support a role for these metabolites as regulators of joint inflammation.
- Detail the association studies (including Mendelian randomization) that have linked low serum 25-hydroxyvitamin D with autoimmune rheumatoid disorders.
- Determine the efficacy of animal models in defining the impact of vitamin D on rheumatoid arthritis.
- Outline the randomized clinical trials that have explored the possible disease prevention and therapeutic benefits of vitamin D supplementation in rheumatoid arthritis.

[2]. RA pathophysiology appears to involve potential underlying genetic susceptibility [3,4] that is further exacerbated by environmental risk factors [5], resulting in the dysregulation of innate and adaptive immunity, and predisposition toward inflammatory autoimmunity rather than immune tolerance [6]. Like other autoimmune diseases documented in this book (see Chapter 97, Chapters 100–102, and Chapter 104), vitamin D deficiency (low serum 25-hydroxyvitamin D, 25(OH)D) has been proposed as a key environmental/dietary factor linked to the pathophysiology of RA [7]. In addition to the relationship between serum 25(OH)D concentrations and RA disease risk and severity, there is now also a well-documented mechanistic rationale for antiinflammatory effects of vitamin D that supports a beneficial function for vitamin D in RA patients. These two facets of vitamin D and RA are discussed in further detail in the following sections, along with the supplementation trials that have investigated the potential benefits of vitamin D supplementation in either preventing or managing RA disease.

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory/autoimmune form of arthritis characterized by synovitis of peripheral joints, with some extraarticular manifestations [1]. If left untreated, ongoing inflammation can result in the destruction of joint tissue, leading to a loss of joint function and potential disability. RA may also lead to premature mortality due, in part, to chronic inflammation promoting adverse cardiovascular health

## 2. Antiinflammatory effects of vitamin D

The interaction between vitamin D and innate and adaptive immunity is described in detail in Chapters 94–96. The central feature of these immunomodulatory responses of vitamin D is the ability of immune cells to metabolize 25(OH)D to active 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) to support both intracrine and paracrine



immunomodulatory actions of  $1,25(\text{OH})_2\text{D}$ . As detailed earlier in Chapter 9, expression of the enzyme 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase) by antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) is stimulated by damage- or pathogen-associated molecular patterns (DAMPs and PAMPs) acting via pattern recognition receptors (PRRs) such as the Toll-like receptor (TLR) system [8,9]. The net effect of this is to actively enhance localized concentrations of  $1,25(\text{OH})_2\text{D}$  within the immediate immune microenvironment. This, in turn, facilitates endogenous intracrine antibacterial (see Chapter 94) and antiviral (see Chapter 95) innate immune activity via vitamin D receptor (VDR)–mediated transcriptional responses [10]. However, localized synthesis of  $1,25(\text{OH})_2\text{D}$  is also central to inflammatory adaptive immune responses, as described in the following sections.

## 2.1 Antigen-presenting cells and extrarenal synthesis of $1,25(\text{OH})_2\text{D}$

One of the initial observations linking vitamin D and immune function was the expression of VDR by APCs such as macrophages [11,12] and DCs [13], and the ability of these cells to therefore respond to  $1,25(\text{OH})_2\text{D}$ . In particular,  $1,25(\text{OH})_2\text{D}$  and its synthetic analogs have been shown to suppress the maturation of DC, leading to attenuation of DC-induced T cell function [14]. This provided the first evidence of a tolerogenic role for  $1,25(\text{OH})_2\text{D}$  mediated via indirect effects on APC rather than direct effects on activated T cells, which unlike resting T cells are known to express VDR [15]. Subsequent studies using monocyte-derived DCs showed that the tolerogenic effects of  $1,25(\text{OH})_2\text{D}$  were associated with suppression of CD80, CD83, CD40, and class 2 major histocompatibility complex (MHC II) and increased apoptosis of mature DC [16,17]. CD4+ T cells activated in the presence of  $1,25(\text{OH})_2\text{D}$ -treated DC showed lower expression of the inflammatory cytokine interferon  $\gamma$  (IFN $\gamma$ ) underlining the indirect antiinflammatory effects of  $1,25(\text{OH})_2\text{D}$ .

Although both macrophages and DC express VDR and respond to  $1,25(\text{OH})_2\text{D}$ , the increased capacity of these cells for synthesis of  $1,25(\text{OH})_2\text{D}$  from  $25(\text{OH})\text{D}$  as they differentiate has highlighted a potential avenue by which  $25(\text{OH})\text{D}$  may also be able to influence immune function [18–20]. Studies using monocyte-derived DCs in coculture with T cells have shown that contact with T cells is sufficient to induce expression of the 1 $\alpha$ -OHase gene (*CYP27B1*) by DC [21]. The resulting DC 1 $\alpha$ -OHase activity enabled localized DC synthesis of  $1,25(\text{OH})_2\text{D}$  that was as effective as exogenous  $1,25(\text{OH})_2\text{D}$  in promoting antiinflammatory T cell responses in the cocultures. This intracrine action of

endogenously generated  $1,25(\text{OH})_2\text{D}$  by DC may be sufficient to modulate inflammatory T helper (Th) cell responses and also to generate tolerogenic regulatory T cells (Treg) within tissues such as the joints.

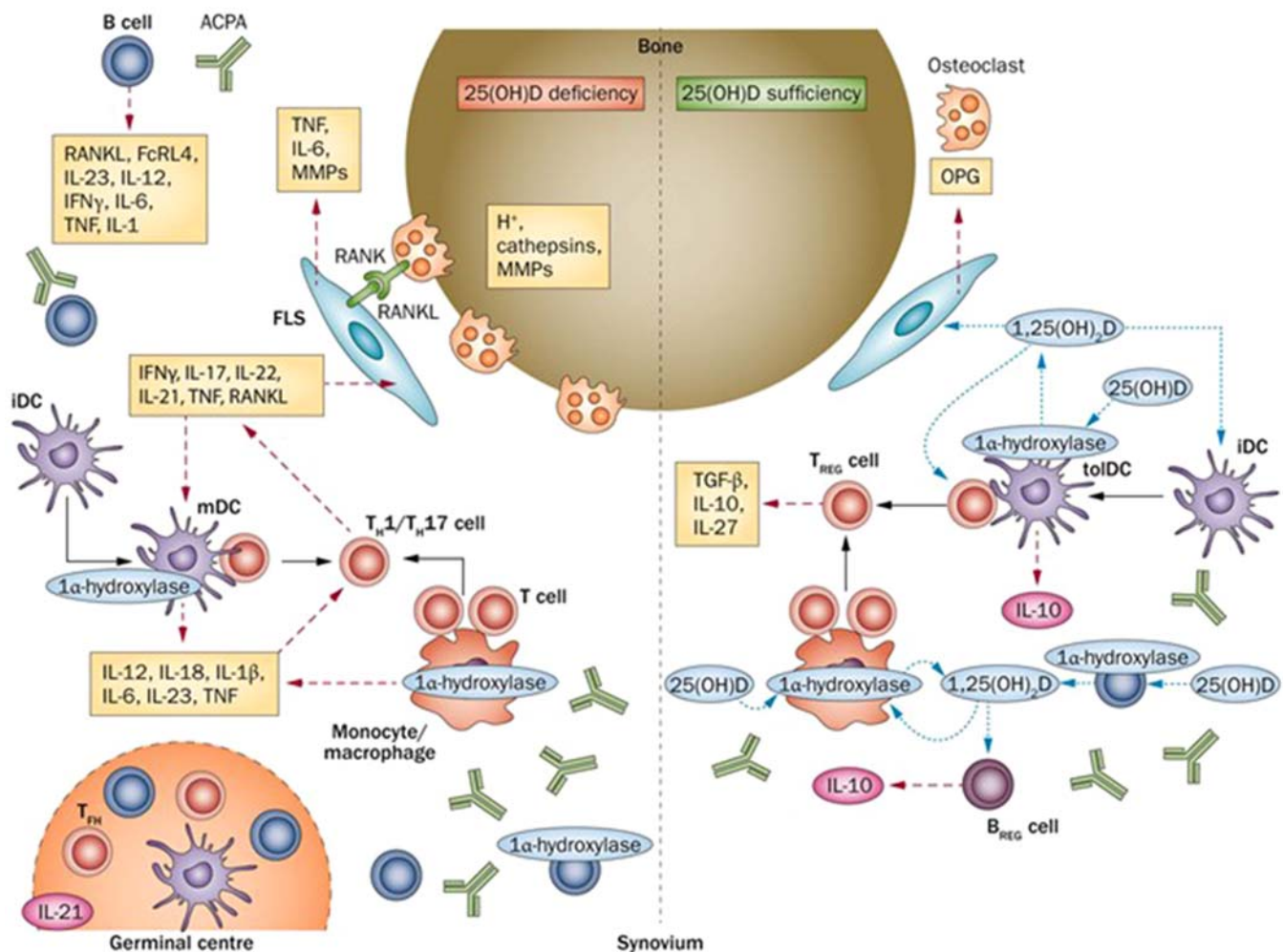
As shown in Fig. 103.1, a key intracrine action of vitamin D is to promote the generation of tolerogenic DCs (tolDCs) that are instrumental in directing tolerogenic T cell responses (IL-10 production and Treg generation). A similar intracrine mechanism may also support Treg generation via macrophages, as well as promoting regulatory B cells (Breg), which, like Treg, play a pivotal role in tolerogenic immune responses during inflammation [22]. The model in Fig. 103.1 shows how DC synthesis by DC is effective in promoting antiinflammatory/tolerogenic T cell responses in the setting of adequate access to  $25(\text{OH})\text{D}$  for metabolism to  $1,25(\text{OH})_2\text{D}$ , in other words under conditions of vitamin D sufficiency. Fig. 103.1 also illustrates the potential consequences of impaired APC synthesis of  $1,25(\text{OH})_2\text{D}$  in the setting of vitamin D deficiency, with mature DC (mDC) predominating instead of tolDC, and mDC and macrophages promoting an inflammatory cytokine milieu and inflammatory Th1/Th17 T cells rather than Treg and Breg.

The overarching message from this hypothetical model system is that healthy (sufficient) serum levels of  $25(\text{OH})\text{D}$  may play a crucial role in suppressing inflammatory immune responses associated with autoimmune diseases such as RA. In this model, the pivotal effects of  $25(\text{OH})\text{D}$  are assumed to be mediated through its metabolism to  $1,25(\text{OH})_2\text{D}$  by APC and subsequent intracrine effects on these cells that indirectly influence T and B cell function. However, it is important to recognize that the synthesis of  $1,25(\text{OH})_2\text{D}$  by APC may also have paracrine effects within the immune microenvironment [8]. In particular, the differential expression of VDR and 1 $\alpha$ -OHase by DC as they differentiate suggests that more active synthesis of  $1,25(\text{OH})_2\text{D}$  by mDC expressing lower levels of VDR may facilitate paracrine effects of  $1,25(\text{OH})_2\text{D}$  on immature DCs (iDCs), which are known to have lower levels of 1 $\alpha$ -OHase but higher levels of VDR [23].

## 2.2 Helper, cytotoxic, and regulatory T cells

The actions of vitamin D in regulating adaptive immune responses via effects on Th and Treg T cell subsets are described in greater detail in Chapter 96.

$1,25(\text{OH})_2\text{D}$  plays an important role in regulating T cell phenotype [24–27]. In particular,  $1,25(\text{OH})_2\text{D}$  has been shown to suppress inflammatory interleukin-17 expressing Th17 cells [28] and Th1 cells [29], while promoting Th2 cells [30] and Treg [31,32].  $1,25(\text{OH})_2\text{D}$  can also promote immune activity by enhancing effector



**FIGURE 103.1 Vitamin D status and synovial immune cell function.** In RA, vitamin D deficiency favors inflammatory responses and osteoclast-mediated bone loss. Autoantigens such as citrullinated matrix proteins are presented to  $CD4^+$  T cells by professional APCs such as DCs or monocytes and macrophages. DCs, monocytes, macrophages, and B cells express the vitamin D-activating enzyme  $1\alpha$ -hydroxylase ( $1\alpha$ -OHase) and can generate  $1,25(OH)_2D$  from  $25(OH)D$  under conditions of vitamin D sufficiency. Although expression of  $1\alpha$ -OHase is enhanced in mDCs, these cells promote a  $T_H1$  cell or  $T_H17$  cell phenotype in the absence of sufficient  $25(OH)D$ .  $IFN\gamma^+$   $T_H1$  cells promote APC maturation, whereas  $T_H17$  cells act on FLSs to promote release of inflammatory cytokines and MMPs and to express RANKL. RANKL expression by FLSs,  $T_H17$  cells, and  $FCRL4^+$  B cells drives osteoclastogenesis and bone erosion. Vitamin D deficiency also favors the production of IL-21 and generation of  $T_{FH}$ -mediated germinal centers for B cell activation and differentiation. By contrast, in conditions of vitamin D sufficiency,  $25(OH)D$  is metabolized to  $1,25(OH)_2D$  by  $1\alpha$ -OHase-expressing APCs, promoting the differentiation of tolDCs, which favor the differentiation of  $T_{REG}$  cells and  $B_{REG}$  cells.  $1,25(OH)_2D$  produced by APCs or  $B_{REG}$  cells might also act directly on T cells and B cells to promote an antiinflammatory phenotype, and enhances expression of OPG over RANKL by FLSs, thereby reducing osteoclastogenesis. Cell differentiation pathways (black arrows), cytokine production and function (red arrows), and vitamin D metabolism (blue arrows).  $1,25(OH)_2D$ , 1,25-dihydroxyvitamin D;  $25(OH)D$ , 25-hydroxyvitamin D; ACPA, anticitrullinated protein antibody; APC, antigen-presenting cell;  $B_{REG}$ , B regulatory cell; DC, dendritic cell;  $FCRL4$ , Fc receptor-like protein 4; FLS, fibroblast-like synoviocyte; iDC, immature dendritic cell; mDC, myeloid dendritic cell; MMP, matrix metalloproteinase; OPG, osteoprotegerin; RA, rheumatoid arthritis; RANK, tumor necrosis factor receptor superfamily member 11A; RANKL, tumor necrosis factor ligand superfamily member 11;  $T_{FH}$ , T follicular helper cell;  $T_H$ , T helper cell; tolDC, tolerogenic dendritic cell;  $T_{REG}$ , T regulatory cell. Reproduced with permission from *Nature Reviews in Rheumatology*, Springer Nature [7].

T cell responses including cytotoxic function [25], via potential effects to promote T cell receptor expression and T cell activation [33], and by modulating natural killer (NK) cells [34]. Effects of  $1,25(OH)_2D$  have also been described for natural killer T (NKT) cells, and NKT cell development is impaired in mice lacking the VDR gene [35].

Studies using activated peripheral blood mononuclear cells (PBMC)-derived  $Th17$  cells from treatment-naïve patients with early RA have shown that treatment with  $1,25(OH)_2D$  reduces expression of IL-17 and  $IFN\gamma$  in a similar fashion to effects of another antiinflammatory steroid, dexamethasone [36]. By contrast in this study,  $1,25(OH)_2D$  stimulated expression of IL-4,

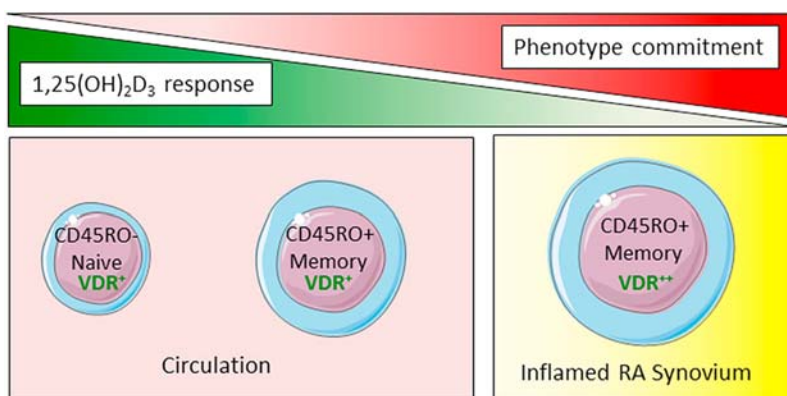
whereas IL-4 was suppressed by dexamethasone. As a consequence, the IL-4:IL-17 ratio, which is elevated in early RA patients, was decreased with  $1,25(\text{OH})_2\text{D}$  but increased with dexamethasone [36]. The conclusion from these studies was that  $1,25(\text{OH})_2\text{D}$  as a treatment for RA may be more effective and have less detrimental side effects than more traditional antiinflammatory therapeutics such as glucocorticoids. Other groups have reported similar effects of  $1,25(\text{OH})_2\text{D}$  in suppressing inflammatory IL-1, IL-6, IL-17, IL-22, and TNF $\alpha$  in T cells from RA patients while simultaneously promoting IL-4 [37]. Recent studies of RA patients receiving the IL-6 receptor antibody tocilizumab showed better response to this treatment when patients were vitamin D sufficient [38]. Analysis in vitro of PBMC-derived T cells from these patients showed that tocilizumab and  $1,25(\text{OH})_2\text{D}$  had a synergistic effect in suppressing Th17 cells [38].

Chronic synovial inflammation is a hallmark of RA and involves the accumulation of inflammatory T cells within the synovium. However, although  $1,25(\text{OH})_2\text{D}$  has been shown to exert potent effects in suppressing Th1 and Th17 cells using PBMC from RA patients, relatively few studies have used T cells from actual RA patient synovial fluid. Work by our group compared T cell responses to  $1,25(\text{OH})_2\text{D}$  using cells isolated from paired blood and synovial fluid samples [39]. One notable difference between the T cell populations from blood and synovial fluid is that synovial T cells are predominantly memory T cells, which constitute only approximately 50% of circulating T cells [40]. Analysis of T cells from RA patients showed that both naïve (immunologically unchallenged) T cells and memory T cells are able to respond to  $1,25(\text{OH})_2\text{D}$ , but inhibition of inflammatory cytokines was more pronounced for naïve T cells when compared with memory T cells [39]. This lack of responsiveness to  $1,25(\text{OH})_2\text{D}$  was even more pronounced in memory T cells from RA synovial fluid compared with memory T cells from paired blood

samples in the same RA patient. T cell responses to  $1,25(\text{OH})_2\text{D}$  appear to be dependent on the commitment of T cells to a particular phenotype. Conversion of cytokine naïve T cells into inflammatory effector cells is strongly suppressed by  $1,25(\text{OH})_2\text{D}$ . Conversely,  $1,25(\text{OH})_2\text{D}$  is much less effective in suppressing an existing T cell phenotype, despite similar expression of VDR in these cells (Fig. 103.2). T cells from synovial fluid exhibit a more committed phenotype relative to circulating T cells. Consequently,  $1,25(\text{OH})_2\text{D}$  is much less effective in suppressing inflammatory T cells from synovial fluid than from circulating blood, even in cells from the same RA patient. The overall conclusion from these observations is that  $1,25(\text{OH})_2\text{D}$  may be less effective as a treatment for diseases such as RA once the disease has become established. Thus, either higher doses of  $1,25(\text{OH})_2\text{D}$  are needed to counter diseases such as RA or attention should be refocused on the use of vitamin D in disease prevention.

In addition to the accumulation of inflammatory T cells within the synovium, RA is also characterized by the presence of senescent T cells, which are sensitive to activation by self-antigens within the joint [42] and continue to produce proinflammatory cytokines. Serum vitamin D status has been linked to telomere length in leukocytes, and this, in turn, may help reduce the rate of T cell senescence [43]. In support of this proposal, vitamin D supplementation in vivo has been reported to increase telomerase activity in PBMCs [44]. The importance of telomerase and T cell senescence in RA is underlined by the fact that even naïve  $\text{CD4}^+$  T cells from patients with RA have impaired telomerase activity [45], which is independent of RA disease activity and duration [42]. Telomerase deficiency could therefore be a potential preclinical marker for RA [42], and increasing serum  $25(\text{OH})\text{D}$  levels through vitamin D supplementation may help to increase telomerase activity, and thus reduce the risk of developing RA, while potentially also slowing the progression of RA.

**FIGURE 103.2** Memory T cells from synovial fluid are less sensitive to  $1,25(\text{OH})_2\text{D}$  than circulating naïve or memory T cells. Both naïve ( $\text{CD45RO}^-$ ) and memory ( $\text{CD45RO}^+$ ) T cells are able to respond to  $1,25$ -dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}_3$ ), but inhibition of inflammatory cytokines is more pronounced for naïve T cells and becomes less effective upon transition from naïve to memory. Furthermore, although naïve to memory transition accounts for most of the loss in  $1,25(\text{OH})_2\text{D}_3$  responsiveness, memory T cells from synovial fluid (SF) show a further decline in  $1,25(\text{OH})_2\text{D}_3$  sensitivity when compared with circulating memory cells. Adapted from an original figure that is reproduced through an Open Access agreement, Elsevier [41].





## 2.3 B cells

Although most studies of adaptive immune function in autoimmunity have to date focused on inflammatory and regulatory T cell function, there is growing interest in the role that B cells play in diseases such as RA [46]. B cells are able to exert positive effects on inflammation through the production of cytokines and associated effects on the differentiation of inflammatory Th17 cells [47]. B cells are also associated with autoantibody production following plasma cell differentiation [48] and contribute to macrophage differentiation [49]. In common with autoreactive T cells, pathogenic B cells are also a feature of RA [50]. As with T cells, the inflamed synovium in RA is characterized by accumulation of B cells [51], and therapies targeted at B cell depletion have recently been expanded from use with B cell lymphomas to the treatment of autoimmune diseases [52]. However, it is important to recognize that, in diseases such as RA, the benefits of pathogenic B cell depletion are offset by potential detrimental effects of Breg depletion. As shown in Fig. 103.1, Bregs play a key role in maintaining tolerogenic immune responses during inflammation [21], and thus, B cell targeted therapies for RA (and other autoimmune diseases) require a delicate balance between the two opposing features of B cells during inflammation.

In contrast to the detailed characterization of T cell responses to 1,25(OH)<sub>2</sub>D, relatively little is known about effects of 1,25(OH)<sub>2</sub>D on B cells in general but particularly in the setting of RA. B cell responses to 1,25(OH)<sub>2</sub>D may include indirect effects via T cell modulation [53], or direct B cell effects on class switching [54], IL-10 production [55] and CCR10 production [56]. Although specific effects of vitamin D on Breg function have yet to be described, the established effects of 1,25(OH)<sub>2</sub>D on B cell IL-10 production [55], and the inverse relationship between IL-10-producing B cells and RA disease activity [57] suggests that 1,25(OH)<sub>2</sub>D may act to support Breg function during inflammation. In a murine pregnancy and asthma model, serum vitamin D deficiency was associated with decreased levels of both Treg and Breg in conjunction with increased levels of inflammatory cytokines such as IL-17 [58]. It therefore seems likely that in autoimmune diseases such as RA, vitamin D will act to promote not only Tregs, but also Bregs. As shown in Fig. 103.1, vitamin D deficiency may therefore also act to impair Breg function and thus predispose to autoimmunity.

## 2.4 Fibroblasts

In addition to innate and adaptive immune cells involved in inflammatory activity, the synovial tissue of joints affected by RA is also characterized by the

presence of fibroblast-like synoviocytes (FLSs) [59]. Initially, FLSs were considered as simply the platform for immune cell interactions [60]. However, it is now clear that FLSs play an active role in the pathogenesis of RA [61,62], and also mediate joint destruction by secretion of cartilage-degrading matrix metalloproteinases, cathepsins, and RANKL (which promotes osteoclastogenesis) and Dickkopf-related protein 1, which inhibits osteoblast function) [61]. Cultured FLSs treated with 1,25(OH)<sub>2</sub>D show decreased levels of inflammatory cytokines and MMPs [63], suggesting that active vitamin can attenuate FLS function. More recent studies have shown that 1,25(OH)<sub>2</sub>D inhibits the viability and promotes the apoptosis of FLS from RA patients [64]. This action is mediated in part by cooperation with DBP, with lower levels of this protein in the synovial tissue and synovial fluid of RA patients [64]. The overall conclusion from these studies was that DBP enhances the effects of 1,25(OH)<sub>2</sub>D on FLS viability and apoptosis. In contrast to these FLS suppressive effects, 1,25(OH)<sub>2</sub>D has been shown to increase RANKL expression in FLS [65]. Although this suggests that 1,25(OH)<sub>2</sub>D promotes osteoclastogenesis and the potential bone loss associated with this, 1,25(OH)<sub>2</sub>D also stimulated FLS production of the RANKL decoy receptor osteoprotegerin (OPG) to significantly increase the OPG/RANKL ratio [65]. In this way, FLS responses to 1,25(OH)<sub>2</sub>D within the synovium may act to decrease the abundance of osteoclasts and thus help to reduce the pathological bone loss associated with RA (see Fig. 103.1). In common with APC within the immune system, FLSs from the synovium of RA patients expressing 1 $\alpha$ -OHase are able to synthesize 1,25(OH)<sub>2</sub>D from 25(OH)D [66,67], thus providing a mechanism by which vitamin D status (serum 25(OH)D levels) can influence FLS function.

## 3. Vitamin D and mouse models of RA

The association between vitamin D and autoimmune disease such as RA has been explored using animal models. This includes analysis of mice under conditions of 25(OH)D deficiency, and/or following supplementation with vitamin D or active 1,25(OH)<sub>2</sub>D. Likewise, mice with knockout of the *Vdr* and *Cyp27b1* genes have provided important insights into the potential causative impact of vitamin D deficiency on RA disease.

Initial studies using two models of murine RA disease, Lyme arthritis and collagen-induced arthritis, induced by infection with *Borrelia burgdorferi* or injection with type II collagen, respectively, showed that supplementation with 1,25(OH)<sub>2</sub>D (20 ng/day) reduced the symptoms of disease associated with both of these murine models of RA [68]. This included scoring of ankle and paw inflammation in living animals and tissue



analysis of joints following euthanasia. Subsequent studies suggested that the effect of supplementary 1,25(OH)<sub>2</sub>D in protecting against murine forms of RA (as well as murine multiple sclerosis) is due, in part, to the induction of interleukin-4 (IL-4) [69]. However, in a more recent study of 1,25(OH)<sub>2</sub>D-mediated protection against collagen-induced arthritis, amelioration of RA disease severity in mice was associated with downregulation of interferon- $\gamma$  (IFN $\gamma$ ) and interleukin-17 (IL-17)-expressing T cells [70]. Conversely, treatment with 1,25(OH)<sub>2</sub>D enhanced expression of Treg cells, indicating that the protective effect of vitamin D on murine RA involves both antiinflammatory and tolerogenic effects. These effects of 1,25(OH)<sub>2</sub>D were also shown to be mediated, in part, by induction of microRNA 124 [70].

In common with other autoimmune diseases, initial murine studies of RA and vitamin D have focused primarily on the impact of supplemental oral 1,25(OH)<sub>2</sub>D on effector T cell or Treg expression and function as a conduit for ameliorating tissue inflammation. However, an alternative strategy is to target tolerance via actions on APCs such as DCs. One such study utilized subcutaneous administration of liposomes encapsulating ovalbumin and 1,25(OH)<sub>2</sub>D to reduce MHC class II expression in DC from draining lymph nodes and thus promote Treg expression and function [71]. The net effect of this was to reduce RA disease severity in the mice by suppression of antigen-specific memory T cell differentiation and function.

In contrast to human studies, vitamin D supplementation in mouse models of autoimmune disease has primarily involved treatment with 1,25(OH)<sub>2</sub>D rather than conventional vitamin D supplementation used for human studies. In most cases, this strategy ameliorated the specific disease, suggesting that simply raising circulating 1,25(OH)<sub>2</sub>D is sufficient to modulate inflammatory disease in a specific animal model. This contradicts the fundamental localized metabolism of 25(OH)D to 1,25(OH)<sub>2</sub>D defined in human studies of inflammation [8] and suggests that the intracrine 25(OH)D model of metabolism may not be generalizable to animal models *in vivo*. It should also be recognized that hypercalcemic effects of 1,25(OH)<sub>2</sub>D may be less evident in animal models of inflammation because of the relatively short-term treatments used in this setting relative to scenarios of human clinical therapy. Thus, extrapolation of animal models of vitamin D and inflammatory disease must be viewed with some caution.

In common with studies of dietary vitamin D deficiency, murine knockout of vitamin D genes such *Vdr* and *Cyp27b1* has been shown to exacerbate mouse versions of autoimmune disease. It therefore seems likely that the vitamin D system plays some role in mediating normal immune responses that protect against inflammatory disease. The first study that illustrated this involved

crossing *Vdr* knockout mice with the human tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) transgenic mouse (hTNFtg) [72]. The resulting cross-bred offspring showed increased clinical symptoms of inflammatory arthritis and increased numbers of macrophage in inflamed joints, suggesting that expression of VDR is central to normal macrophage antiinflammatory function in hTNFtg mice [72]. In addition, macrophages from the *Vdr* knockout/hTNFtg mice showed increased potential to differentiate into bone-resorbing osteoclasts, suggesting that VDR expression may also be important in limiting the cartilage destruction and bone erosion associated with synovial inflammation. Exacerbation of experimental inflammatory arthritis has also been observed in mice lacking the 1 $\alpha$ -hydroxylase enzyme. Knockout mice for *Cyp27b1* subjected to collagen-induced arthritis showed increased clinical evidence of joint inflammation, as well as elevated rheumatoid factor and C-reactive peptide [73]. Intraperitoneal administration of 1,25(OH)<sub>2</sub>D (1  $\mu$ g/kg every other day) rescued the effects of *Cyp27b1* knockout by restoring serum 1,25(OH)<sub>2</sub>D levels to wild-type levels [73]. In addition to showing that loss of circulating 1,25(OH)<sub>2</sub>D in *Cyp27b1* knockout mice was associated with increased levels of inflammatory cytokines, the authors also noted hyperplasia of FLSs and showed that this was countered by peritoneal administration of 1,25(OH)<sub>2</sub>D, which decreased FLS proliferation while also stimulating apoptosis of these cells [73]. As with studies of 1,25(OH)<sub>2</sub>D administration in other mouse models of RA, the overarching conclusion from the observations of *Cyp27b1* knockout mice is that enhanced circulating levels of 1,25(OH)<sub>2</sub>D alone may be sufficient to ameliorate key features of inflammatory arthritis, at least in mouse models of this disease.

#### 4. Vitamin D deficiency and RA disease risk, severity, and progression

In common with many other human health conditions detailed in this section of the book, a crucial observation linking vitamin D and RA has been the association between low serum 25(OH)D status and either the incidence or disease severity of RA. Systematic reviews have reported a relationship between serum 25(OH)D and clinical and laboratory indices of RA disease activity [74,75]. Although these metaanalyses identified important differences between the studies included in the systematic reviews, both studies reported lower levels of serum 25(OH)D in RA patients versus healthy controls, and both also reported an inverse relationship between serum 25(OH)D and RA disease activity. A selection of the most prominent reported studies of serum 25(OH)D levels in patients with RA is shown in Table 103.1.

**TABLE 103.1** Association between serum vitamin D levels and RA disease activity.

Study References	Population size and location	Disease duration	Analytical method	Metabolite measured	25(OH)D deficiency	25(OH)D lower in HC versus RA	Association of vitamin D with disease parameter(s)
[76]	RA = 96, Germany	Mean 12.2years (range 6 m–to 38years)	RAI	1,25(OH) <sub>2</sub> D	Unclear	n/a	Neg: disease activity Pos: urinary collagen cross-links ↑ DA is assoc. neg. Ca balance and ↓ bone formation
[77]	RA = 118, HC = 75, Estonia and Italy	Not stated	RAI	25(OH)D	n/a	n/a	Neg: DAS-28; however, correlation varied according to time of year and country of origin
[78]	RA = 266 (African American)	Mean 31.2m (SD = 7.3m)	Unclear	25(OH)D	<15 ng/mL	n/a	Null
[79]	RA = 62, United States	Mean 11.6years (SD = 12.3years)	Quest Lab-Corp	25(OH)D	<30 ng/mL	n/a	Neg: DAS28, pain and HAQ in active RA (DAS28 > 2.6) only
[80]	RA = 1191, HC = 1019, Italy	Mean 11.5years (SD = 8.7years)	ELISA	25(OH)D	<30 ng/mL	No	Neg: HAQ disability, DAS28, MADLS, high Steinbrocker functional state
[81]	Rheumatic disease = 121 (RA = 85), Israel	Mean = 9.9years (SD = 8.5years)	NOS	25(OH)D	Unclear	n/a	Null
[82]	RA = 65, HC = 40, Turkey	Mean = 7.73–7.95yrs	EIA	25(OH)D	Not specified	No	Neg: DAS-28, CRP, HAQ
[83]	RA = 44, HC = 44, Greece	Not stated	RAI	25(OH)D <sub>3</sub>	n/a	Yes	Neg: DAS-28, CRP, ESR
[84]	RA = 499, United States, China	Not stated	ELISA	25(OH)D	<50 nmol/L (<30 ng/mL)	Yes	Null
[85]	RA = 100, HC = 100, Saudi Arabia	Mean 4.7years (SD = 5years)	LC MS/MS	25(OH)D	<30 and < 10 ng/mL	No	Neg: DAS28 Study used two definitions for 25(OH)D deficiency
[86]	RA = 55, HC = 45, Turkey	Not stated	Elecsys	25(OH)D	<30 nmol/L	Yes	Null

*Continued*

TABLE 103.1 Association between serum vitamin D levels and RA disease activity.—cont'd

Study References	Population size and location	Disease duration	Analytical method	Metabolite measured	25(OH)D deficiency	25(OH)D lower in HC versus RA	Association of vitamin D with disease parameter(s)
[87]	RA = 108, UIA = 39, HC = 239, Iran	Not stated	ELISA	25(OH)D	<20 ng/mL	No	Correlation of 25(OH)D with RA disease parameters was not an objective of this study; the study simply compared 25(OH)D between disease/control
[88]	RA = 55, PsA = 43, HC = 40, Egypt	Mean 4.93years (SD = 3.11years)	CLA	25(OH)D	n/a	Yes	Null
[89]	RA = 110, HC = 110, China	Mean = 6.51years, SD = 6.82years	RAI	25(OH)D	Not specified	n/a	Neg: DAS28
[90]	RA = 4793, Japanese	Mean = 12years	RAI	25(OH)D	<20 ng/mL	n/a	Neg: Japanese HAQ disability score, NSAID use
[91]	RA = 302, Denmark	Mean = 10.5years (range = 0–50years)	HPLC-MS	25(OH)D	<50 nmol/L (<30 ng/mL)	n/a	No assoc. overall but <15 nmol/L 25(OH)D associated with increased DAS28 > 5.1, CRP, RF and ≥3 DMARDs
[92]	RA = 126, New Zealand	Mean = 12years (range 1–37years)	Immuno-assay NOS	25(OH)D	<50 nmol/L (<30 ng/mL)	n/a	Neg: VAS. This parameter of the DAS28 score alone accounted for assoc. with RA
[93]	Rheum dx = 56 (RA = 39), nonrheum dx = 60	Not stated	NOS	25(OH)D	<50 nmol/L (<30 ng/mL)	Yes	Neg: DAS28-ESR
[94]	RA = 71, AS = 72, OA = 74, HC = 70, Turkey	Not stated	HPLC	25(OH)D	n/a	No	Null
[95]	RA = 120, HC = 1341, United States	Not stated	RAI or LC MS/MS	25(OH)D	<20 ng/mL + <30 ng/mL	n/a	Null assoc. between 25(OH)D and RA onset
[96]	RA = 63, HC = 62, Egypt	Mean = 5.89years (SD = 3.67years)	CLA	25(OH)D	<20 ng/mL	Yes	Neg: QoL, HAQ II, FMS RA + FMS had lower 25(OH)D than RA alone
[97]	RA = 130, HC = 80	Mean = 6years (range 2m–40years)	ELISA	25(OH)D	n/a	Yes	Neg: SJC, TJC, joint pain, EMS, HAQ, Plt, ESR, IL-17, IL-23

[98]	Pre-RA = 166, HC = 490	n/a	RAI	25(OH)D	n/a	n/a	Null association between 25(OH)D and development of RA, except in a small subset of females just prior to RA onset
[99]	RA = 99, HC = 68, Iran	Mean = 59years (SD 5.6years; range 0.2 –20years)	ELISA	25(OH)D	<30 nmol/L	No	Null—however, all patients were on Vit D replacement
[100]	RA = 80, HC = 80	Not stated	ELISA	25(OH)D	<10 ng/mL	Yes	Neg: DAS28
[101]	RA = 73, UA = 40, OA = 58, NIA = 89, other IA = 50, ReA = 14, CrA = 19	RA–49years (range 18–88)	Not stated	25(OH)D	n/a	No	Null
[102]	RA = 97, OA = 28, Poland	5.8 ± 5.4years (25(OH) D > 20 ng/dL) 8.8 ± 9.8years (25(OH) D < 20 ng/dL)	CLA	25(OH)D	<20 ng/dL	n/a	Neg: DAS28, HAQ, BDI Pos: PA, SF-36
[103]	RA = 181, HC = 186, Japan	Mean = 10.2years (5.2 –20years)	RAI	25(OH)D	Not specified	Yes	Null
[104]	RA = 102, Saudi	Not stated	CLA	25(OH)D	<30 ng/mL	n/a	Neg: DAS28
[105]	RA = 34, HC = 41, Argentina	Mean = 7.6years (SD = 1.4years)	CLA	25(OH)D	<20 ng/mL (<50 nmol/L)	Yes	Neg: DAS-28
[106]	RA = 116, China	Not stated	ELISA	25(OH)D	<50 nmol/L (<20 ng/mL)	Yes	Null
[107]	Early RA = 154, HC = 60, China	Disease duration <1year	CLA	25(OH)D	<20 ng/mL	Yes	Neg: ACPA, ESR, DAS
[108]	RA = 894, HC = 861, multiple countries	Not available	NOS	25(OH)D	≤10 ng/mL	Yes	Neg: DAS28-CRP, SDAI, CDAI
[109]	RA = 239, Thai	Median = 84m (range = 48–132m)	CLA	25(OH)D <sub>2</sub> 25(OH)D <sub>3</sub>	n/a	n/a	Null
[110]	RA = 66, Iran	Not stated	CLA	25(OH)D	n/a	n/a	Neg: DAS-ESR, SJC, TJC, GHS, EMS
[111]	RA = 100, HC = 50	Not stated	CLA	25(OH)D	n/a	Yes	Neg: TNF-α, IL-1β, IL-6, IL-10, IL-17, ROS
[112]	RA = 1413, 15 countries	8.3years (range 3.6 –15.2years)	NOS	25(OH)D	≤10 ng/mL	n/a	Neg: DAS + corticosteroid dose

Continued



TABLE 103.1 Association between serum vitamin D levels and RA disease activity.—cont'd

Study References	Population size and location	Disease duration	Analytical method	Metabolite measured	25(OH)D deficiency	25(OH)D lower in HC versus RA	Association of vitamin D with disease parameter(s)
[113]	RA = 625, HC = 276, 13 European countries	Mean = 11years (SD = 9years)	CLA	25(OH)D	<20 ng/mL	Yes	Neg: DAS28-CRP, RAID, HAQ, SRS/HRS/GRS domains of D-PRO
[114]	RA = 160, Denmark	Median = 14.1 weeks (range = 6.1–26.6)	LC MS/MS RAI	25(OH)D <sub>2</sub> 25(OH)D <sub>3</sub> 1,25(OH) <sub>2</sub> D	<50 nmol/L	n/a	Null Null Neg: DAS28-CRP, HAQ, CRP, VAS-pain Pos: ACPA
[115]	RA = 100, Ecuador	Full article in Spanish	Unclear	25(OH)D	Spanish	n/a	Null
[116]	RA = 41, HC = 41	Not available	Unclear	Unclear	Unclear	Yes	Neg: PROs
[117]	RA = 161, China	Mean = 79m (range 3–360m)	Immunoassay	25(OH)D	Unclear	n/a	Lower serum 25(OH)D in RA patients with depression (higher HAMD and HAMA scores). Also higher DAS28-ESR, VAS, and HAQ with depression
[118]	RA = 50, HC = 50, India	Not available	ELISA	25(OH)D	<30 ng/mL	Yes	Neg: DAS28
[119]	RA = 280, HC = 140, China	Mean = 9years (SD = 9years)	ELISA	1,25(OH) <sub>2</sub> D	<25 ng/mL	Yes	Neg: DAS28 and ESR, Th17 Pos: Treg
[120]	RA = 20, reactive RA = 7, HC = 23, United Kingdom	Range = 7 weeks–to 18years	LC-MS/MS (serum and SF)	25(OH)D <sub>2/3</sub> 1,25(OH) <sub>2</sub> D <sub>3</sub> epi25(OH)D <sub>3</sub> 24,25(OH) <sub>2</sub> D <sub>3</sub>	n/a	No	Neg: SJC (25(OH)D <sub>3</sub> and epi25(OH)D <sub>3</sub> ) Pos: SJC and CRP (25(OH)D <sub>2</sub> )
[121]	RA = 90, HC = 30, Iran	Not available	HPLC	25(OH)D	Unclear	Yes	Neg: DAS28, ESR
[122]	RA = 169, Norway	Median = 9years (range 4–17.5years)	LC-MS/MS	25(OH)D <sub>2</sub> /D <sub>3</sub>	<30 nmol/L	n/a	Null: fatigue
[123]	RA = 160, Denmark	Approximately 1year	LC-MS/MS RIA	25(OH)D <sub>2</sub> /D <sub>3</sub> 1,25(OH) <sub>2</sub> D <sub>3</sub>	<50 nmol/L unclear	n/a	Pos: adjusted DAS28 remission, season of diagnosis
[124]	RA = 368, UK	Median symptoms = 24.1 days (range 13.0–52.2 days)	LC-MS/MS	25(OH)D <sub>3</sub>	Unclear	n/a	Null: ESR, CRP, TJC68, SJC66, TJC28, SJC28, DAS28-ESR, DAS28-CRP, VAS pain, VAS EMS

[125]	RA = 156, Japan	Mean disease duration = 16.1years SD 12.7	ELI	25(OH)D	Low = 5.9–16.0 ng/mL High = 16.1–32.1 ng/mL	n/a	Neg: HAQ, sarcopenia, severe sarcopenia Pos: skeletal mass index, hand grip strength, gait speed
[126]	RA = 300, HC = 300, Pakistan	Not available	Unclear	25(OH)D	<30 ng/mL	Yes	Neg: rheumatoid factor
[127]	RA = 188, HC = 158, China	Not available	ELI	25(OH)D	<20 ng/mL	Yes	Sarcopenia and low 25(OH)D may be risk factors for the incidence of vertebral fractures in RA patients

*ACPA*, anticitrullinated peptide antibody; *CDAI*, Clinical Disease Activity Index; *CLA*, chemiluminescent assay; *DA*, disease activity; *DAS28*, disease activity score 28; *ELI*, electrochemiluminescence immunoassay; *ELISA*, enzyme-linked immunosorbent assay; *EMS*, early morning stiffness; *FMS*, fibromyalgia syndrome; *GHS*, Global Health Score; *GRS*, Global Risk Score (SRS + HRS); *HAQ*, Health Assessment Questionnaire; *HC*, healthy control; *HRS*, Habitus Risk Score; *HPLC*, high-performance liquid chromatography; *LC MS/MS*, liquid chromatography–tandem mass spectrometry; *Neg*, negative correlation between vitamin D and outcome measure; *NOS*, not otherwise specified; *OA*, osteoarthritis; *Pos*, positive correlation between vitamin D and outcome measure; *PROs*, patient reported outcomes; *PsA*, psoriatic arthritis; *RA*, rheumatoid arthritis; *RIA*, radioimmunoassay; *ROS*, reactive oxygen species; *SDAI*, Simple Disease Activity Index; *SJC*, swollen joint count; *SRS*, symptom risk score; *TCJ*, tender joint count; *VAS*, visual analogous score.

There is considerable heterogeneity with the studies presented in this table, with regard to geographical location, methodology for measuring circulating 25(OH)D, and the precise definition of vitamin D deficiency in each report. Moreover, the majority of listed studies are observational or cross-sectional in nature, and thus a causal role for vitamin D in RA cannot necessarily be inferred. Nevertheless, the prevalence of vitamin D deficiency in RA patients and negative correlation between serum 25(OH)D and various markers of RA disease strongly suggest that vitamin D has some impact on RA disease. This is illustrated by recent systematic reviews of the association between vitamin D and RA. A metaanalysis of 15 studies of vitamin D status and RA using 1143 patients and 963 controls showed that serum 25(OH)D deficiency was more common in RA patients (55.2%) relative to healthy controls (33.2%) [74]. Further analysis of 13 of these studies (including 924 RA patients) showed a significant inverse correlation between serum 25(OH)D concentration and RA disease activity as defined by RA disease activity score 28 joints (DAS28) [74]. Another metaanalysis published at the same time as the study described before reported similar data using 24 published reports and 3489 RA patients also showed lower levels of serum 25(OH)D in RA patients and correlation between 25(OH)D and DAS28 [75]. The conclusion from both of these studies is that serum 25(OH)D concentration is associated with both the risk of developing RA and subsequent disease severity.

Increased RA onset, severity, and progression has been reported for winter months, consistent with the decline in serum 25(OH)D levels that is observed following peak UVB-induced levels of 25(OH)D in later summer [128]. In common with many other diseases that have been linked to vitamin D deficiency, low serum 25(OH)D is not necessarily a causative factor in RA. It is possible that severe RA may impair serum 25(OH)D levels by limiting patient access to UV-mediated synthesis of vitamin D. Inflammatory autoimmune disease may also act to suppress serum 25(OH)D levels either at the level of renal clearance of 25(OH)D via decreased availability of vitamin D-binding protein (DBP), or through an as-yet-unidentified effect on metabolism of vitamin D to 25D. It is also important to recognize that vitamin D deficiency may impact RA beyond the central pathophysiological effects on inflammatory joint damage. Low serum levels of 25(OH)D may contribute to established comorbidities of RA such as the bone disease osteoporosis [129]. Vitamin D deficiency is one of the key risk factors for osteoporosis and potential bone fractures in RA patients [89,97,105,127,130], and this is further exacerbated by the widespread therapeutic use of glucocorticoids in RA patients; a major contributor to bone loss in its own right [131]. Thus, the use of

antiinflammatory steroids against the backdrop of vitamin D deficiency may be a worst-case scenario for potential bone loss in RA patients.

Another comorbidity of RA that is likely to be affected by vitamin D status is the pain associated with the inflammatory disease. Pain features strongly in the RA disease parameters described in Table 103.1, but may persist even when the disease is in remission. Several studies have specifically explored the impact of vitamin D on this facet of RA disease. A multicenter cross-sectional study of 93 patients visiting outpatient rheumatology clinics showed that neuropathic pain was 5.8 times more prevalent among patients with serum 25(OH)D levels less than 20 ng/mL compared with those with 25(OH)D levels that were higher than 30 ng/mL [132]. The overarching conclusion from this study was that serum 25(OH)D concentrations were a good predictor of likely neuropathic pain in RA patients. Other studies have reported that supplementation with the active form of vitamin D, 1,25(OH)<sub>2</sub>D (60,000 IU once weekly in combination with 1000 mg/day calcium carbonate), improves pain relief [133]. In 150 treatment-naïve early RA (<2 years) subjects attending rheumatology clinic, visual analog scale (VAS) and disease activity score-28 days (DAS-28) were significantly improved in the 1,25(OH)<sub>2</sub>D-treated subjects compared with the calcium-only group [133]. A link between vitamin D and RA disease pain has also been described for fibromyalgia, a chronic disorder associated with severe pain that is one of the most common rheumatological conditions in patients presenting at rheumatology clinics [134]. Supplementation with vitamin D (bolus dose of 100,000 IU followed by 4000 IU/day for 16 weeks) showed improved chronic back pain vitamin D-deficient (<30 ng/mL serum 25(OH)D at baseline) or overweight adults who were otherwise healthy [135]. Similar results were also observed for a cohort of 68 patients with chronic lower back pain who were treated with 60,000 IU/week vitamin D for 8 weeks [136]. In a similar fashion to that observed for other human health conditions, the effect of vitamin D supplementation in managing pain appears to be most pronounced in patients with low serum 25(OH)D at baseline, providing another potential advantage in assessing vitamin D status in RA patients.

One approach to resolve the issue of whether vitamin D deficiency is a causative factor in RA is through the use of vitamin D supplementation randomized control trials to determine if elevation of serum 25(OH)D levels can prevent the onset or progression of RA disease. This strategy is discussed in greater detail in Section 5 and Table 103.2 of this chapter. Further insight into the possible benefits of vitamin D in preventing RA has also been provided by food fortification programs (see Chapter 58). Reported studies of a large cohort of

TABLE 103.2 Vitamin D supplementation trials in rheumatoid arthritis.

Study	Study participants (eligible number of RA patients + DMARD tx)	Treatment groups/trial design/BL 25(OH)D	Primary and secondary outcome measures	Summary of key findings
[137]	RA = 19 (on MTX ± GC, active dx)	2 microg/day oral alphacalcidol for 3/12 in 2 groups; mod + highly active RA. Control group = same patients data collected over 3 months prior to suppl. Open-label trial	ESR, CRP, EMS, Richie index, Lee index at 3 months	CRP, SJC, TJC, Richie index, and Lee index all significantly decreased after 3/12. RF and CRP were decreased, but this was not statistically significant.
[138]	RA = 121 (on triple DMARDs)	500 IU 1,25(OH) <sub>2</sub> D <sub>3</sub> + CaCO <sub>3</sub> versus CaCO <sub>3</sub> Open-label 25(OH)D < 20 ng/mL at BL	Pain relief assessed by patient VAS at first relief of pain and again at 3/12	No difference in time in achieving first pain relief; however, there was higher pain relief in the 1,25(OH) <sub>2</sub> D <sub>3</sub> group at 3/12 (NNT = 5).
[139]	RA = 117 (on MTX ± HCQ/CQ, active dx)	50,000 IU/week vitamin D <sub>3</sub> for 3 months versus placebo Double-blinded trial	>0.6 or >1.2 improvement in DAS28 at week 12	No improvement in outcome measures reported.
[140]	RA = 80 (remission for 2/12)	Vitamin D <sub>3</sub> 50,000IU/week versus placebo Double-blinded RCT 25(OH)D levels were <30 ng/mL at BL	DAS28 as a marker of relapse, over 6/12	No statistically significant reduction in relapse rate was observed.
[141]	RA = 22 (on DMARDs)	25(OH)D < 25 ng/mL at BL placebo or vitamin D2 50,000 IU ×3 times/week for 4 weeks, then 50,000 IU ×2/month for 11 months	Primary = serum PTH Secondary = BMD, DAS28, HAQ, and SF36 scores and cytokines	No improvement in PTH, BMD, RA disease activity or cytokines
[142]	RA = 377 (RA in remission)	Alfacalcidol 0.25 microg BD for 24 months in 25(OH)D def. RA versus placebo versus RA with normal 25(OH)D levels and no treatment Open-label Deficiency = 25(OH)D < 30 ng/mL	VAS, SHC, TJC, CRP, ESR, and DAS28 every 2–3/12	Normal vitamin D assoc. with ↓ recurrence. No difference was observed with or without vitamin D suppl. In RA with low vitamin D.
[143]	Early RA = 39 (Tx naïve), HC = 31	MTX + GC versus MTX + GC + 300,000 IU vitamin D (one-off dose) Double-blind RCT	T Cell phenotypes, OC precursors, inflammatory cytokines, clinical parameters at 3/12	Reduced IL-23, increased GlobalHealth Score in vitamin D group
[144]	RA = 73 (on DMARDs, active dx)	60,000 IU/vitamin D <sub>3</sub> /week for 6 weeks then 60,000 IU/month for	Improvement in DAS28-CRP, vitamin D status	↓ DAS28-CRP and ↑ vitamin D > 20 ng/mL in the tx group

Continued



TABLE 103.2 Vitamin D supplementation trials in rheumatoid arthritis.—cont'd

Study	Study participants (eligible number of RA patients + DMARD tx)	Treatment groups/trial design/BL 25(OH)D	Primary and secondary outcome measures	Summary of key findings
[145]	RA = 59 (active disease, DAS28 > 2.6, 17 yrs disease)	3/12 Open-label 25(OH)D < 20 ng/mL at BL + DAS28-CRP > 2.6  Double-blinded RCT, 100,000 IU vitamin D <sub>3</sub> or placebo 25(OH)D < 30 ng/mL at BL	Primary = HAQ; secondary = improvement in DAS28ESR, DAS28CRP, ESR, CRP, RAID score, fatigue (EVA and FACIT), and SF36	HAQ scores improved in vitamin D arm Only ESR and CRP improved in secondary markers
[146]	RA = 108 (active disease mean 82 months, on DMARDs)	Serum 25(OH)D mean 22 ng/mL (43% < 20 ng/mL) Vitamin D3 100,000 IU/month	DAS28CRP, VAS, serum 25(OH)D	VAS higher in 25(OH)D deficiency Supplementation ↓ VAS only in 25(OH)D deficiency
[133]	RA = 150 (treatment-naïve early RA < 2 years)	60,000 IU 1,25(OH) <sub>2</sub> D <sub>3</sub> /week + CaCO <sub>3</sub> carbonate (1000 mg/day), placebo = CaCO <sub>3</sub>	Primary = time for pain relief and VAS Secondary = change in DAS28	↓ VAS and DAS28 scores but no effect on time to pain relief
[147]	RA = 2534 (all received DMARDs —MTX, HCQ, LEF)	4.4 ± 4.9 months. Varied. 1,25(OH) <sub>2</sub> D <sub>3</sub> (0.25–0.5 µg/day), or CaCO <sub>3</sub> and vitamin D (200–400 IU/day)	Primary = % patients with moderate/good response to RA treatment at 3, 6, 12, and >12 month Secondary = disease activity (VAS, SHC, TJC, CRP, ESR, and DAS28)	No difference in primary or secondary outcomes
[148]	25,871 healthy subjects, >50 years old, no RA	Vitamin D <sub>3</sub> (2000 IU/day) or placebo, and omega 3 fatty acids (1000 mg/day) or placebo, or both. For median 5.3 years	Primary = all incident self-reported autoimmune disease. Followed by medical confirmation Secondary = individual autoimmune diseases	Vitamin D with or without omega 3 fatty acids reduced autoimmune disease by 22% 40% decrease in RA with vitamin D (only 40 reported cases)

BD, twice daily; BL, baseline; CaCO<sub>3</sub>, calcium carbonate; CQ, chloroquine; CRP, C-reactive protein; DAS, disease activity score; DAS28, DAS 28 joints; DMARDs, disease-modifying antirheumatic drugs; EMS, early morning stiffness; ESR, erythrocyte sedimentation rate; GCs, glucocorticoids; GHS, Global Health Score; HAQ, Health Assessment Questionnaire Disability Index; HCQ, hydroxychloroquine; LEF, leflunomide; MTX, methotrexate; NNT, number needed to treat; RA, rheumatoid arthritis; RAID score, rheumatoid arthritis impact of disease; RF, rheumatoid factor; SF36, 36-Item Short Form Health Survey; SJC, swollen joint count; TJC, tender joint count; Tx, treatment; VAS, visual analogue scale.

patients with rheumatic diseases from Finland, where fortification of foods with vitamin D has been routine for 20 years, showed that vitamin D deficiency was relatively low in RA patients from this particular country [149]. Thus, it is important to recognize that there may be significant geographical variations when assessing associations between vitamin D and RA, and when planning supplementation trials, particularly in countries where fortification of food is common. However, some insight into the potential causative effects of vitamin D status on RA can also be derived from studies of the genetics of vitamin D metabolism and function, and this is discussed in greater detail in [Section 4](#).

## 5. Genetic variation in the vitamin D system and RA

Genetic analysis of links between the vitamin D system and RA initially focused on single nucleotide polymorphisms (SNPs) in the *VDR* gene and included analysis of RA disease risk and severity. However, in view of the well-established link between vitamin D and bone disease, *VDR* gene variants have also been studied with respect to bone loss in RA patients. Early studies of the *VDR* gene using 120 Spanish patients with RA showed weak association of *Bsm1* and *Taq1* SNPs with early-onset RA in female patients [150]. In a similar study from France using families with RA and healthy controls, *Fok1* polymorphisms were associated with RA [151]. Since these initial publications, there have been many subsequent studies of *VDR* SNPs and RA, using geographically distinct cohorts. A recent systematic review of these publications utilized 23 eligible studies to conclude *Fok1* was protective against RA in general but particularly in Asians and Europeans [152]. In African and Arabic cohorts, *Taq1* decreased RA but showed no effect in the overall analysis. Contrary to earlier studies, *Bsm1* did not appear to associate with RA in this metaanalysis. The overarching conclusions from this collection of studies was that genetic variations with the *VDR* were linked to RA disease risk, although there was significant geographical variation in which SNPs were informative. Furthermore, in common with many other studies of *VDR* gene variations and human disease, it is still unclear what specific 1,25(OH)<sub>2</sub>D signaling mechanism is affected by the SNPs associated with RA.

In addition to assessing the impact of *VDR* polymorphisms on risk of RA, several studies have also investigated possible effects on RA disease severity and associated complications. In a study of 123 patients from Spain with RA, the *bb* genotype of *Bsm1* was associated with less severe disease [153]. In the context of the *VDR*, the most commonly studied complication of RA is

bone loss. *Taq1* SNPs have also been shown to be associated with bone loss in women [154,155]. Similar observations have also been made in a cohort of male and female RA patients from Hungary [156]. However, a study in a Korean cohort showed no relationship between *VDR* SNPs and RA-associated bone erosion [157]. Similarly, a study in a German cohort of 62 RA patients and 40 healthy controls showed no association between *VDR* SNPs and markers of bone turnover [158].

Although the *VDR* gene has been the most widely studied with respect to effects on RA risk and disease severity and complications, genetic variations in other components of the vitamin D system have also been assessed in patients with RA. Prominent among these are variations in the gene for DBP (GC). In one study, proteomic and mass spectrometry analysis of synovial tissue from 10 patients with RA highlighted possible involvement of DBP in RA, with lower levels of DBP detected in tissue from these patients [159]. Subsequent GC genotyping in a larger group of RA patients and healthy controls showed that GC SNPs were associated with RA disease risk [159]. In another study of 1957 RA patients from Japan, a GC SNP was shown to be associated with lower serum levels of 25(OH)D and also increased hip fracture risk in the RA patients [160]. Genetic variations in genes for two vitamin D metabolism enzymes, 25-hydroxylase (*CYP2R1*) and *CYP27B1*, have been shown to be associated with increased risk of RA [161]. Although SNPs in *VDR* and *CYP24A1* were not associated with RA in this study, methylation levels of *CYP27B1* and *VDR* were both linked to RA disease risk [161].

The overarching message from studies of individual SNPs is that genetic variations in genes from the vitamin D system may influence both the serum levels of 25(OH)D in RA patients and risk of RA disease itself. As detailed in Chapter 60, various SNPs in the vitamin D system contribute to the genetic component of circulating 25(OH)D levels. This, in turn, has enabled prediction of the genetic component of serum 25(OH)D, based on combinations of SNPs associated with the handling and metabolism of vitamin D, which can be used in Mendelian randomization (MR) analyses to assess the possible impact of serum 25(OH)D on disease risk. The advantage of this approach is that it predicts a possible lifetime exposure to higher or lower serum 25(OH)D, and thus, any positive association with a disease is more likely to be causative rather than a consequence of the disease (see Chapter 61 for specific details of MR analysis). Initial MR analysis of almost 2000 RA patients did not support an effect of low predicted serum 25(OH)D on RA disease risk [162]. A subsequent study of patients with RA or systemic lupus erythematosus reported similar conclusions [163]. However, most vitamin D MR analyses to date have assumed a linear

relationship between genetically determined serum 25(OH)D and disease risk or severity. The recent observation of a nonlinear association between genetically determined 25(OH)D for overall mortality [164] and cardiovascular disease [165] in a large cohort of UK Biobank samples is consistent with a threshold effect where disease risk was significantly higher for those individuals with serum 25(OH)D less than 50 nmol/L. It will be interesting in future studies to see if there is a similar nonlinear threshold effect for genetically predicted serum 25(OH)D and RA.

## 6. Effects of supplementary vitamin D on RA

In common with other extraskeletal diseases where association studies have linked to vitamin D deficiency, there have been far fewer studies that have investigated possible beneficial effects of vitamin D supplementation on RA disease. This is due in part to the inherent difficulties in executing vitamin D supplementation trials (choosing target population, daily versus bolus dosing, choice of dosing, and compliance with supplementation regime are all potential confounders). However, as with other autoimmune disease, the possible use of vitamin D supplementation has the additional complication of *when* supplementation is most effective. As can be seen from sections earlier in this chapter, mechanistically it is likely that vitamin D is most effective in *preventing* the onset of autoimmune disease. However, any supplementation trial to address this would therefore require treatment of tens of thousands of healthy subjects to determine if the relatively small number of those subjects who develop autoimmune disease is diminished in those receiving vitamin D. This is particularly relevant if assessing the effects of vitamin D on disease risk for a specific autoimmune disorder such as RA, despite the fact that this is one of the more common autoimmune diseases. In this setting, the period of vitamin D supplementation to allow sufficient individuals to develop autoimmune disease and statistically assess possible effects of vitamin supplementation would be many years. Even if this was logistically possible, there are still likely to be complications with compliance and monitoring the effects of supplementation with respect to serum vitamin D status. It is therefore not surprising that, to date, most studies of vitamin D supplementation and RA have focused on investigating the effects of vitamin D supplementation on disease severity.

Table 103.2 shows a summary of the most prominent vitamin D supplementation trials in patients with RA. This illustrates the heterogeneity of the supplementation studies that have been reported so far. Some studies used early RA patients, others with sustained active

disease, and others with patients in remission. In some cases, patients were treatment naïve, while others involved patients receiving disease-modifying antirheumatic drugs (DMARDs). In a similar fashion, the mode of supplementation with vitamin D also varied. Weekly bolus dosing with 50,000 IU vitamin D<sub>3</sub> or higher doses was common, but other forms of vitamin D such as 1,25(OH)<sub>2</sub>D or alphacalcidol were also employed. Out of 12 studies shown in Table 103.2 that investigated effects of vitamin D supplementation, only 5 reported significant effects on disease severity, and this involved a variety of RA disease scores or markers. A 2020 systematic review of six studies from Table 103.2 [138,139,141,143,145,146] reported an overall significant improvement in DAS28, erythrocyte sedimentation rate (ESR), and tender joint score (TJC) with vitamin D supplementation but not for other markers of RA disease [166]. In selected subgroups and treatment durations, vitamin D supplementation did improve other markers of RA, and the overall conclusion was that vitamin D was an effective intervention for patients with RA [166]. It is also worth noting that in recent dietary recommendations, the French Society for Rheumatology stated that there was no indication for vitamin D (and other vitamins) for controlling the activity of chronic inflammatory rheumatic disease [167].

Of the 13 studies in Table 103.2, only 1 study to date has addressed the question of whether or not vitamin D supplementation provides protection against the onset of RA. The Vitamin D and Omega 3 Trial (VITAL) is a large-scale (25,871 subjects) vitamin D and omega fatty acid double-blind, placebo-controlled supplementation trial that was established to assess the effects of vitamin D<sub>3</sub> (2000 IU/day) and marine omega 3 fatty acids (1 g/day fish oil capsule with 460 mg eicosapentaenoic acid and 380 mg docosahexaenoic acid) in preventing cancer and cardiovascular disease [168] (see Chapter 78 for further details on this trial). A 5-year follow-up of VITAL showed a significant decrease in confirmed and probable autoimmune diseases in the vitamin D supplemented group, particularly after the initial 2 years of follow-up were excluded [148]. This observation was unaffected by cotreatment with omega fatty acids. The strengths of this study are the large number of participants and the extended period of supplementation adherence. However, a weakness of the study is that the number of subjects who developed autoimmune disease was small, and this prevented clear observations for individual autoimmune diseases. Nevertheless, for specific diagnosis of RA, there was a 40% reduction in those receiving vitamin D supplementation (15 versus 24 with RA from 12,927 to 12,944 subjects, respectively), and these data may improve with further follow-up [148]. Analysis of serum 25(OH)D levels in subsets of the trial participants showed an

increase from 29.8 ng/mL at baseline to 41.8 ng/mL after 1 year of vitamin D supplementation confirming that the supplementation regimen was sufficient to elevate serum vitamin D status. However, it is unclear if these elevated levels are maintained across the 5-year follow-up. More importantly, baseline levels of 25(OH)D of approximately 75 nmol/L indicate that the trial cohort was vitamin D sufficient even in the absence of the trial supplement (those in the placebo arm were allowed to continue routine vitamin D supplementation if less than 800 IU/day) [148]. It is tempting to speculate that a similar supplementation trial in participants with serum 25(OH)D levels less than 50 nmol/L at baseline might result in more significant effects in preventing autoimmune disease and RA in particular.

## 7. Conclusions

In common with other autoimmune diseases, there is a strong mechanistic link between vitamin D and the inflammation associated with RA. The synovial fluid and tissue of the inflamed joints of RA patients comprise an array of cells that can both synthesize 1,25(OH)<sub>2</sub>D from 25(OH)D, providing a link between vitamin D status (serum 25(OH)D levels) and the potential availability of 1,25(OH)<sub>2</sub>D within disease-affected tissues. The ability of this 1,25(OH)<sub>2</sub>D to direct a range of antiinflammatory, tolerogenic, and bone-sparing responses with these tissues means that vitamin D has the potential to act as a versatile suppressor of RA. Multiple association studies have endorsed this mechanistic rationale for vitamin D and RA by showing that low levels of serum 25(OH)D are associated with increased RA disease risk and severity. However, this is not necessarily evidence of a causative role for vitamin D deficiency in RA. Until recently, vitamin D supplementation trials had not shown any significant benefit in RA. However, the majority of these studies were focused on the impact of serum 25(OH)D levels on disease progression in established RA cases. Recent data from the VITAL supplementation trial suggest that enhanced 25(OH)D may be more effective in preventing RA. Bigger and much longer-term studies are needed to confirm the apparent protective effect of vitamin D in preventing diseases such as RA, but it also has to be recognized that the optimal serum level of 25(OH)D for good “immune health” (and prevention of autoimmune diseases) is still unclear. The potential benefits of vitamin D for existing RA patients also require a rethink. Can vitamin D be used alongside existing RA therapies? This possible strategy was endorsed by studies of the CD28 ligand blocker abatacept, which acts to block T cell activation in RA. Experiments in vitro showed that cotreatment with 1,25(OH)<sub>2</sub>D enhanced the efficacy of abatacept,

suggesting that vitamin D supplementation may be a useful adjunct for existing RA therapies [169]. It will be important for future studies to build on the existing wealth of information of vitamin D metabolism and function within the immune system to provide a better framework for the use of vitamin D in RA.

## 8. Summary points

- Intracrine and paracrine actions of 1,25(OH)<sub>2</sub>D by cells from the innate and adaptive immune system and by synovial fibroblasts are associated with potent systemic and synovial antiinflammatory responses.
- The T cell actions of 1,25(OH)<sub>2</sub>D are diminished in the memory T cells that predominate in the synovial fluid of joints.
- Low serum 25(OH)D is associated with increased risk of RA and increased risk of RA disease progression.
- Vitamin D supplementation trials have shown conflicting effects on RA disease risk and severity. However, recent data from the VITAL trial suggest a protective effect on risk of autoimmunity in older adults.

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## Further reading

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# Psoriasis and other skin disorders

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## OBJECTIVES

- To review the history of the use of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub>, and its analogs in dermatology.
- To provide the reader with an understanding of how 1,25-dihydroxyvitamin D<sub>3</sub> and its analogs can affect keratinocyte proliferation and differentiation.
- To review the physiology of the complex network regarding vitamin D metabolism and signaling in the skin.
- To review the clinical utility of 1,25-dihydroxyvitamin D<sub>3</sub> and its analogs for the treatment of psoriasis.
- To provide the reader with up-to-date information on the use of 1,25-dihydroxyvitamin D<sub>3</sub> and its active analogs for treating other autoimmune and hyperproliferative skin disorders.

## 1. Introduction/historical overview

Earlier in the past century, vitamin D<sub>3</sub> was used in dermatology in huge pharmacological doses for the treatment of scleroderma, psoriasis, lupus vulgaris, and atopic dermatitis. A rationale for the use of vitamin D in psoriasis was the clinical observation that this disease, in general, markedly improves in the summer. In 1936, J. Krafka wrote in *The Journal of Laboratory and Clinical Medicine* under the headline “A simple treatment for

psoriasis”: “A commonly observed fact in the South concerning this disease (psoriasis) is that it generally clears up in the summer sun. This led the author to the hypothesis that it might be cured with viosterol; irradiated ergosterol containing vitamin D<sub>2</sub>. A psoriasis patient with a case of 10 years standing, continuous duration was put on viosterol. Within 60 days from the beginning of the treatment, the skin of the patient was entirely clear” [1]. A year later, Ceder and Zon [2] reported 15 cases of widespread chronic psoriasis, 3 of whom had whole-body involvement. They were protected from sunlight as much as possible, and each patient received either 300,000–400,000 IUs of irradiated ergosterol or pure crystalline vitamin D<sub>2</sub> in sesame oil (50,000 IUs/capsule) orally daily. 10 of the 12 subjects who took the vitamin D<sub>2</sub> in sesame oil showed complete involution of their psoriasis within 6–12 weeks. Of the three patients, who took the irradiated ergosterol preparation, one showed complete involution and the other two obtained partial improvement within 10 weeks. All of the patients, except one, developed hypercalcemia ranging from 12 to 16 mg/dL. In 1950, Spier [3] reported results of a study with 94 psoriasis patients performed in 1948–49. First, patients received orally 3 × 10 mg vitamin D<sub>2</sub>/week for 2–4 weeks, thereafter 2 × 10 mg vitamin D<sub>2</sub>/week until a total dose of ~300 mg vitamin D<sub>2</sub> was reached. In this study, patients were treated for 3–4 month; 20% of patients showed a good response, 25% a satisfactory response, 25% a moderate response, and 30% a nonsatisfactory response [3].

But these first attempts of vitamin D treatment in dermatology were abandoned because of severe vitamin D intoxication that caused hypercalcemia, hypercalciuria, and kidney stones occurred when these huge pharmacological doses of vitamin D (up to 1000-fold of the

regular daily requirement of vitamin D) were used, and because other new treatments, including corticosteroids and retinoids, were introduced for the therapy of these diseases.

## 2. Pathogenesis of psoriasis

### 2.1 Psoriasis: Pathogenesis, immunology, and histology of skin lesions

Psoriasis is a chronic dermatosis of unknown etiology characterized by skin inflammation and hyperproliferation and altered differentiation of epidermal keratinocytes [4,5]. The most common form of the disease is plaque psoriasis, in which skin develops scaly, red lesions. The severity of chronic plaque psoriasis ranges from mild, when the disease has only a mild impact on quality of life, to severe, when patients' lives are significantly affected. In severe cases, most of the body surface, including the scalp and nails, may be involved. The peak age of onset for this psychologically debilitating and disfiguring disease is the second decade, but psoriasis may first appear at any age from infancy to the aged [6]. It is considered a multifactorial disease and has a prevalence of about 1%–2% in the United States. Population, family, and twin studies clearly demonstrate that there is a strong but very complex genetic component, leading to the development of psoriatic skin lesions [7]. Most likely, multiple genes are involved in the pathogenesis of psoriasis. During the past years, molecular biology techniques have been developed that allow studies to analyze psoriasis susceptibility genes, but up to today, no specific genetic marker of the disease is identified. Psoriasis has long been known to be associated with certain HLA antigens, particularly HLA-Cw6, although there is no evidence that a psoriasis susceptibility gene exists at this locus [8].

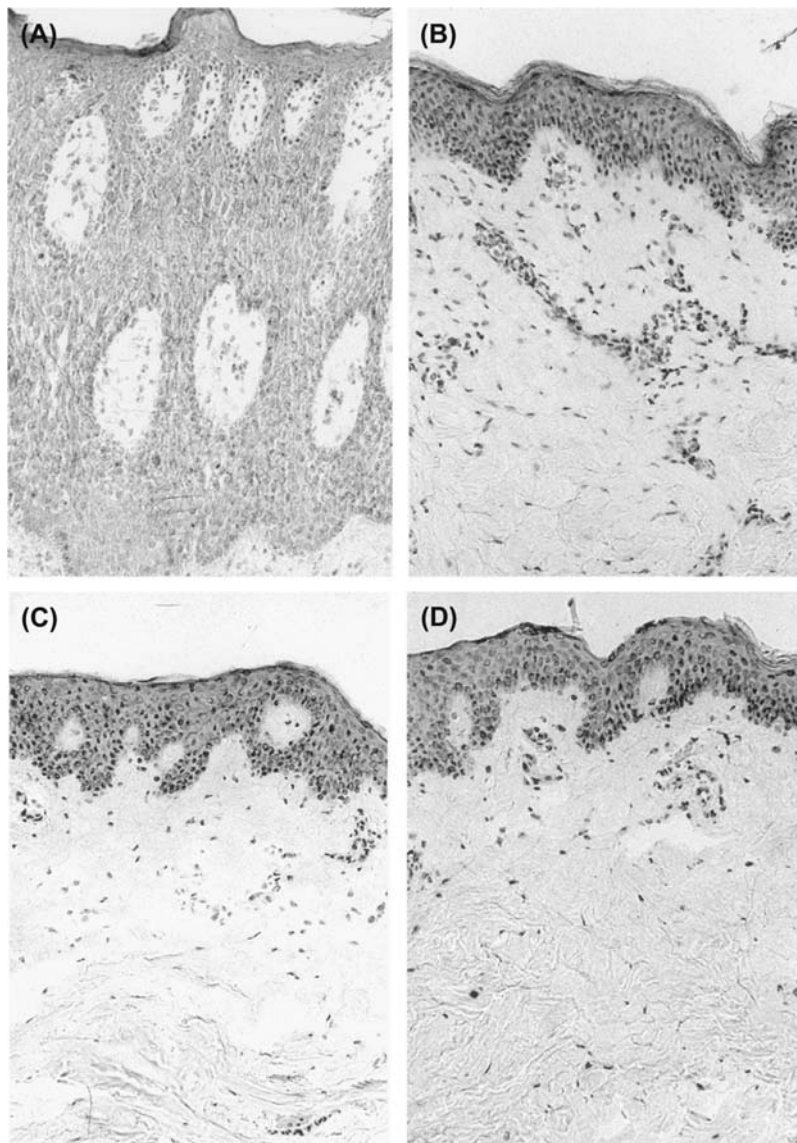
Until today, it is still not finally resolved what cell types in human skin are primarily affected by the disease. Many studies indicate that epidermal hyperproliferation in psoriasis is caused by cells of the immune system, most likely T lymphocytes [4]. The vast majority of T cells in psoriatic lesions are situated in the perivascular area in the dermis; a high number are also found in the epidermis. Activated CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells in psoriatic lesions express HLA-DR, the interleukin (IL)-2 receptor (CD 25), bear the CLA + memory-effector CD45RO + phenotype, and secrete specific immune mediators and cytokines, such as IL-2 and interferon- $\gamma$  [9–46]. Thus, psoriasis represents mainly a so-called T helper 1 (Th1) profile disease (characterized by T lymphocyte secretion of IL-2, IL-12, and interferon- $\gamma$ ) [9]. In contrast, atopic dermatitis represents a so-called

Th2 profile disease that is characterized by T cell secretion of IL-4, IL-5, and IL-10 [10]. The activation signal for the development of psoriatic lesions is still unknown, although there is increasing evidence that superantigens such as the N-terminal component of bacterial M-proteins may be of importance for the initiation of T cell proliferation in psoriasis [4,9,11,47,48]. It has also been hypothesized that psoriasis patients develop an effector immune response to skin (auto) antigens, which are yet to be specifically identified. According to this model, the immunologic process underlying psoriasis begins with a sensitization-type phase during which the skin dendritic cells migrate to regional lymph nodes where they present these skin antigens to naïve T cells. This sensitization phase occurs prior to the development of skin lesions [11]. When sensitization is obtained, the psoriasis skin lesion may develop as a result of the emigration of T cells in the skin where they are activated by antigen-presenting cells (APCs) including Langerhans' cells presenting self-skin antigens [11].

The precise appearances of the histology of the skin will depend on the age of the psoriatic lesion and the site of the biopsy [12]. In general, epidermal hyperplasia, in which the granular layer may be lost, and the stratum corneum shows parakeratosis, can be found (Fig. 104.1). Typical lesions histologically will show elongation of the dermal papillae, with a relatively thin epidermis at the top of the papillae. The epidermis may show, in suprapapillary compartments, intercellular edema, and infiltration with T-lymphocytes and neutrophils, which can extend into spongiform pustules of Kogoj or Munro microabscesses [12].

### 3. The vitamin D system in normal and psoriatic skin

Vitamin D is photochemically synthesized by ultraviolet-B (UV-B) action in the skin [13–17] (also see Chapter 4 [vol. 1 of this book]). Alternatively, vitamin D<sub>2</sub> and D<sub>3</sub> can be supplemented by a balanced diet of sun-dried or UV-B-exposed mushrooms and animal origin including oily fish, cod liver oil, and foods that are fortified with vitamin D, respectively [17]. Many health agencies and scientific organizations highly recommend vitamin D supplementation to maintain bone health and reduce risk for many chronic illnesses including deadly cancers, autoimmune disorders, neurocognitive dysfunction, type 2 diabetes, and infectious diseases [18]. Vitamin D, whether produced in the skin from 7-dehydrocholesterol (7-DHC) or absorbed from the diet, is activated first in the liver to 25(OH)D and then in the kidneys to the classical biologically active metabolite 1,25(OH)<sub>2</sub>D<sub>3</sub> [17]. Notably, recent studies have revealed that a large number of other biologically



**FIGURE 104.1** Histological demonstration of morphological changes in lesional psoriatic skin after 6 weeks of topical treatment with calcitriol (15 µg/g, B) and calcipotriol (50 µg/g, C). (A) Lesional psoriatic skin before treatment. (D) Nonlesional psoriatic skin. Notice strong reduction of epidermal thickness after topical treatment with vitamin D analogs [74]. Hematoxylin–eosin staining. Original magnification  $\times 200$ .

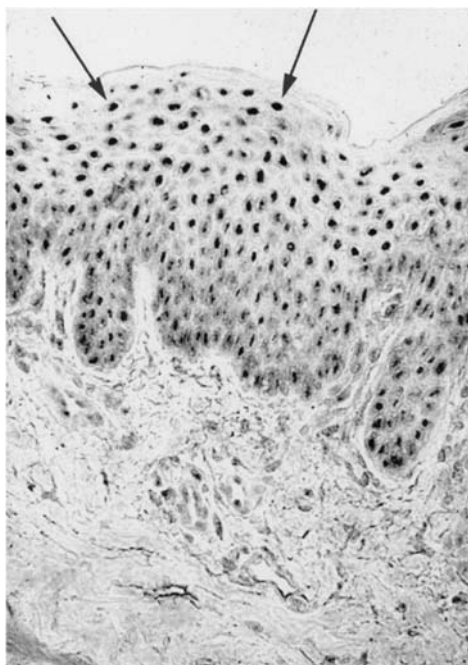
active vitamin D metabolites can be synthesized and activated through a CYP11A1-driven noncanonical metabolic pathway and by other mechanisms [19–25]. It is known that the skin itself is a target tissue for vitamin D compounds, including the classical biologically active secosteroid hormone  $1,25(\text{OH})_2\text{D}_3$  [15–17].  $1,25(\text{OH})_2\text{D}_3$  binds to the vitamin D–binding protein (DBP, GC) in the circulation and is delivered to many target tissues, such as the intestine, bone, and kidneys, to regulate the calcium and phosphate homeostasis [17]. Moreover,  $1,25(\text{OH})_2\text{D}_3$  is also produced locally in many tissues including the epidermis and various immune cells [17], where it exerts autocrine or paracrine effects.

While  $1,25(\text{OH})_2\text{D}_3$  represents the classical biologically active vitamin D metabolite,  $25(\text{OH})\text{D}$  is the major (inactive) circulating form of vitamin D, commonly used as a serological indicator to evaluate the vitamin D status in patients [17,26]. In general, the functions of vitamin D analogs are characterized as genomic, i.e., mediated through vitamin D receptor (VDR)–mediated transcriptional effects inside the cell nucleus, and non-genomic, when VDR induces rapid signaling, including the ability to stimulate calcium transport across the plasma membrane [17,27,28]. To mediate its genomic effects,  $1,25(\text{OH})_2\text{D}_3$  binds to VDR with high affinity ( $K_D$   $10^{-9}$ – $10^{-10}$  M) and low capacity [18,19], which is present in many target tissues, including bone,



gastrointestinal tract, skeletal muscle, or skin [20], as well as in various immune cells, including dendritic cells, monocytes/macrophages, or lymphocytes [17]. Binding of its ligand  $1,25(\text{OH})_2\text{D}_3$  induces conformational changes that enables the VDR to form a heterodimer with retinoid x receptor (RXR) and to bind to vitamin D response elements (VDREs) in the genome to activate or suppress transcription of hundreds of genes in a cell-specific manner [21,22]. Nongenomic effects of  $1,25(\text{OH})_2\text{D}_3$  and analogs are, in part, related to effects on intracellular calcium [23,24]. In keratinocytes and other cell types,  $1,25(\text{OH})_2\text{D}_3$  rapidly increases free cytosolic calcium levels [23,24]. In the skin, both VDR (Fig. 104.2) and retinoid X receptor alpha ( $\text{RXR}\alpha$ ) are expressed in keratinocytes, fibroblasts, Langerhans cells, sebaceous gland cells, endothelial cells, and most cell types related to the skin immune system [17,20,25].

Apart from the classic role of vitamin D in the calcium and phosphate homeostasis, many *in vivo* studies have shown that active vitamin D metabolites additionally regulate a broad variety of other physiological processes, such as cell proliferation, differentiation, and immune modulation. Active vitamin D metabolites display immunomodulatory activity manifested by reduction in the APCs activity or proinflammatory T helper 1 (Th1) and T helper 17 (Th17) frequencies, as well as expansion of the T and B regulatory cells [26–29].



**FIGURE 104.2** Immunohistochemical demonstration of 1,25-dihydroxyvitamin  $\text{D}_3$  receptors (VDRs) in human skin. Notice strong nuclear VDR immunoreactivity in cells of all layers of the viable epidermis (arrows). Labeled avidin–biotin technique using mAb 9A7 $\gamma$  directed against VDR. Original magnification  $\times 400$ .

In addition, it has been reported that CYP11A1-derived vitamin D metabolites serve as ligands for VDR or can act as inverse agonists on retinoic acid receptor–related orphan receptors (ROR)  $\alpha$  and  $\gamma$  [30,31], which are known to play key roles in regulation of many immune and metabolic pathways. More recently, the top signaling pathways for CYP11A1-driven vitamin D analogs, such as  $20,23(\text{OH})_2\text{D}$ , were linked to activation of the aryl hydrocarbon receptor (AhR), and liver X receptors (LXR)  $\alpha$  and  $\beta$ , representing alternative receptors to VDR [32–36]. In this context, it has to be noted that despite the importance of UVB radiation for vitamin  $\text{D}_3$  synthesis, this optical radiation is also a key agent that induces DNA damage in the skin [37]. Both,  $1,25(\text{OH})_2\text{D}_3$  and CYP11A1-derived vitamin D analogs, protect epidermal keratinocytes against UVB-induced damage via activation of the Nrf2-dependent antioxidant response and p53-phosphorylation, as well as by induction of the DNA repair system [38]. In addition, both, the classical  $1,25(\text{OH})_2\text{D}_3$  and noncalcemic CYP11A1-driven analogs, exert antiinflammatory effects on keratinocytes by inhibiting the nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) activity [39,40].

*In vitro* studies have revealed that  $1,25(\text{OH})_2\text{D}_3$  is extremely effective in inducing the terminal differentiation and in inhibiting the proliferation of cultured human keratinocytes in a dose-dependent manner [41–43]. Additionally,  $1,25(\text{OH})_2\text{D}_3$  acts on many cell types involved in immunologic reactions, including lymphocytes, macrophages, and Langerhans cells (see Chapters 94–96). Data about the effects of  $1,25(\text{OH})_2\text{D}_3$  on the melanin pigmentation system are still conflicting, but most studies do not support that  $1,25(\text{OH})_2\text{D}_3$  regulates melanogenesis in human skin [44].

A study indicated that cutaneous mast cells may mediate their immunomodulatory effects at least in part via the VDR. In this investigation, biopsies were collected from the nonlesional and lesional skin of patients with actinic keratosis (AK), Bowen's disease/squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and psoriasis. Expression of the metabolic enzymes, vitamin D-25-hydroxylase (CYP2R1, 25-OHase), and 25-hydroxyvitamin D- $1\alpha$ -hydroxylase (CYP27B1,  $1\alpha$ -OHase) in mast cells were analyzed by immunohistochemistry using a sequential double-staining method. The percentage of mast cells immunoreactive for CYP27A1 was significantly higher in lesional as compared with nonlesional skin in all diseases, especially in SCC and BCC. Moreover, the percentage of mast cells immunoreactive for CYP27B1 was significantly increased in BCC, AK, and psoriatic lesions as well. Only about 5%–6% and 2% of the mast cells expressed CYP27A1 and CYP27B1, respectively, in the nonlesional skin of psoriatic and AK patients. In contrast, 23%–38% and 6%–9% of the mast cells were

immunoreactive for CYP27A1 and CYP27B1, respectively, in the nonlesional skin of BCC and SCC patients. In human mast cell cultures, about 30% and 15% of the mast cells showed CYP27A1 and CYP27B1, respectively. The authors concluded that mast cells may promote an immunosuppressive environment, e.g., in skin carcinoma [45].

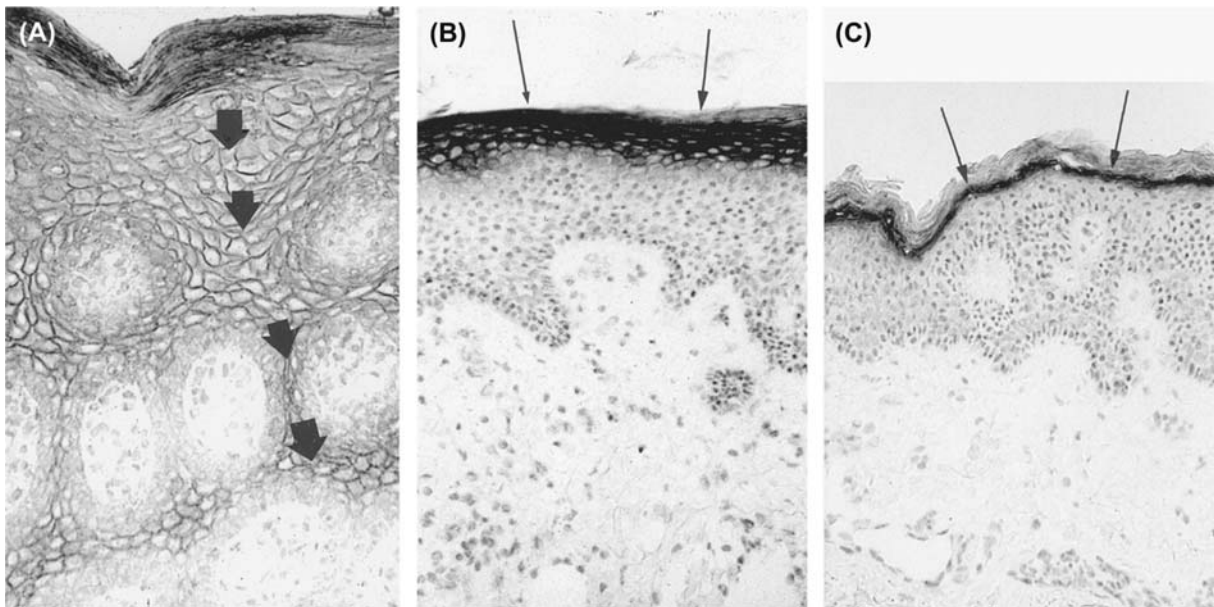
Sebocytes are sebum-producing cells that form the sebaceous glands. When the vitamin D endocrine system (VDES) in human sebocytes was analyzed recently, it was shown that sebocytes represent target cells for biologically active vitamin D metabolites [46]. It was demonstrated that human SZ95 sebocytes express VDR and the enzymatic machinery to synthesize and metabolize biologically active vitamin D analogs [46]. The expression of the VDR and the metabolic enzymes, CYP27A1, CYP27B1, and 1,25(OH)<sub>2</sub>D<sub>3</sub>-24-hydroxylase (CYP24A1, 24-OHase) were all detected in human SZ95 sebocytes in vitro using real-time quantitative polymerase chain reaction [46]. Although several other splice variants of 1 $\alpha$ -OHase were detected by nested touchdown polymerase chain reaction, it was demonstrated that the full-length product represents the major CYP27B1 gene product in SZ95 cells [46]. It was shown that incubation of SZ95 sebocytes with 1,25(OH)<sub>2</sub>D<sub>3</sub> resulted in a cell culture condition-, time-, and dose-dependent modulation of cell proliferation, cell cycle regulation, lipid content, and IL-6/IL-8 secretion in vitro, whereas RNA expression of VDR and

CYP24A1 was upregulated along with vitamin D analog treatment [46]. The authors concluded that the VDES is of high importance for sebocyte function and physiology and that sebaceous glands represent potential targets for therapy with vitamin D analogs or for pharmacological modulation of 1,25(OH)<sub>2</sub>D<sub>3</sub> synthesis and metabolism [46].

#### 4. Physiological and pharmacological actions of vitamin D analogs in normal and psoriatic skin

##### 4.1 Biological effects of vitamin D and analogs in psoriasis

The mechanisms underlying the therapeutic effectiveness of vitamin D analogs in psoriasis are still not completely understood. Results from immunohistochemical and molecular biology studies indicate that the antiproliferative effects of topical 1,25(OH)<sub>2</sub>D<sub>3</sub> on epidermal keratinocytes are more pronounced as compared with effects on dermal inflammation [49]. Modulation of various markers of epidermal proliferation (proliferating cell nuclear antigen and Ki-67 antigen) and differentiation (involucrin, transglutaminase K, filaggrin, cytokeratins 10,16) in lesional psoriatic skin after topical application of vitamin D analogs were shown in situ [49] (Fig. 104.3). Interestingly, effects of topical treatment with vitamin D analogs on dermal



**FIGURE 104.3** Immunohistological detection of transglutaminase K in lesional psoriatic skin before treatment (A), lesional psoriatic skin after 6 weeks of topical treatment with calcipotriol (50 µg/g, B), and in nonlesional psoriatic skin (C). Notice strong staining for transglutaminase K in all epidermal cell layers of lesional psoriatic skin before treatment (A, arrows). In contrast, after 6 weeks of topical treatment with calcipotriol staining in lesional psoriatic skin (B, arrows) is restricted to the upper layers of the viable epidermis, a staining pattern that is characteristic for nonlesional psoriatic skin (C, arrows). Original magnification  $\times 160$ .

inflammation are less pronounced (CD-antigens, cytokines, HLA-DR, etc.) as compared with effects on epidermal proliferation or differentiation. One reason for this observation may be that the bioavailability of this potent hormone in the dermal compartment may be markedly reduced as compared with the epidermal compartment [49].

However, some investigations demonstrate immune modulatory effects that may be of relevance for the therapeutic effectiveness of vitamin D analogs in psoriasis. Applying immunohistochemistry and flow cytometry, a study investigated in psoriasis lesions ( $n = 36$ ) of patients with chronic plaque psoriasis ( $n = 18$ ) the immunomodulatory effect of topical treatment with calcipotriol ( $50 \mu\text{g/g}$  vs. vehicle twice a day). After 14 days of treatment, no differences in the frequency of CD4(+) and CD8(+) T cells or innate lymphoid cells between calcipotriol- and vehicle-treated skin were found. No changes in the frequency of IL-22(+) or IFN- $\gamma$ (+) cells were observed. However, a significant decrease in CD8(+) IL-17(+) T cells in skin-derived cells from calcipotriol-treated skin was found, concomitant with clinical improvement, which was further supported by the absence of CD8(+)IL-17(+) T cells in immunohistochemical staining of calcipotriol-treated skin [50].

Interestingly,  $1,25(\text{OH})_2\text{D}_3$  and analogs have been shown to induce immunologic responses not only in conventional dendritic cells (cDCs) but also in plasmacytoid DCs (pDCs). pDCs comprise a specialized, naturally occurring DC subset known to be important in autoimmune diseases including psoriasis. pDCs from the blood rapidly infiltrate psoriatic skin and may represent a key to the initiation of the immune-mediated pathogenesis of the disease. Recently, it has been demonstrated that pDCs express key proteins of the VDES, including CYP27B1 and CYP24A1, and that VDR is transcriptionally active in pDCs. Moreover, vitamin D signaling impairs the capacity of murine and human pDCs to induce T cell proliferation and secretion of the T-helper 1 cytokine IFN $\gamma$ . This inhibitory effect is dependent on the expression of the VDR in the DCs. The authors concluded that vitamin D signaling can act as a natural inhibitory mechanism on both cDCs and pDCs, which may instigate the development of vitamin D-based therapeutic applications for psoriasis and other inflammatory skin diseases [51]. Some findings indicate that the immunomodulatory properties of vitamin D and analogs are mediated in part through effects on regulatory T cells (Treg). Mattozzi et al. [52] demonstrated an association of vitamin D status in psoriasis patients ( $n = 26$ ) with circulating Treg population ( $P < .001$ ) and with psoriasis area and severity index (PASI) score ( $P = .04$ ). The authors concluded that low serum levels of  $25(\text{OH})\text{D}$  may decrease the number of circulatory Treg, disrupting the immunological homeostasis in

psoriatic patients and encouraging the inflammatory activity [52].

Topically applied vitamin D compounds may induce effects on extracellular matrix (ECM) in psoriatic skin. Hyaluronan (HA), the major ECM component, is often anchored to CD44, a family of structurally/functionally important cell surface receptors. Both large HA polymers and their UVR-induced catabolic products (small HA) selectively activate CD44-mediated cellular signaling in human keratinocytes, with all of the downstream processes that include regulation of proliferation, differentiation, and inflammation being mediated by Rho GTPases (e.g., Rac1 and Rho). Interestingly, it has been reported that  $1,25(\text{OH})_2\text{D}_3$  not only prevents the UVR-induced small HA activation of abnormal keratinocyte behavior but also enhances large HA stimulation of keratinocyte activities and epidermal function(s). It was speculated that matrix HA and its UVR-induced catabolic products (e.g., large and small HA) can selectively activate CD44-mediated cellular signaling such as GTPase (Rac and RhoA) activation [53].

Cubillos et al. [54] investigated mechanisms that may underlie in part the efficacy of active vitamin D compounds in psoriatic arthritis. This study investigated osteoclast differentiation and cytokine secretion of peripheral blood mononuclear cells (PBMCs) from patients with psoriasis vulgaris and psoriatic arthritis, in response to  $1,25(\text{OH})_2\text{D}_3$ . Psoriatic arthritis patients had lower osteocalcin, as well as higher C-telopeptide of type I collagen and cathepsin K serum levels compared with psoriasis vulgaris patients and controls. Receptor activator of nuclear factor kappa-b ligand/macrophage-colony stimulating factor-stimulated PBMCs from psoriatic arthritis patients produced higher proinflammatory cytokine levels and had a differential secretion profile in response to  $1,25(\text{OH})_2\text{D}_3$ , compared with psoriasis vulgaris and control PBMCs. Interestingly,  $1,25(\text{OH})_2\text{D}_3$  abrogated altered bone turnover in psoriatic arthritis patients, and increased osteoclastogenic potential and proinflammatory cytokine secretion capacity of their PBMCs compared with psoriasis vulgaris and controls.

Molecular biology studies have demonstrated that clinical improvement in psoriatic lesions treated topically with  $1,25(\text{OH})_2\text{D}_3$  (calcitriol) correlates with an elevation of VDR mRNA [55]. It is well known that some patients suffering from psoriasis are resistant to topical  $1,25(\text{OH})_2\text{D}_3$  treatment. It was demonstrated that responders can be distinguished from the nonresponders at the molecular level because nonresponders show no elevation of VDR mRNA in skin lesions along with the treatment and express relatively low levels of VDR. These data suggest that the ability of calcitriol to regulate keratinocyte growth is closely linked to the expression of VDR. The target genes of topical



1,25(OH)<sub>2</sub>D<sub>3</sub> that are responsible for its therapeutic efficacy in psoriasis are still unknown. Major candidates for 1,25(OH)<sub>2</sub>D<sub>3</sub> target genes that are responsible for the 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced terminal differentiation in keratinocytes are distinct cell cycle-associated proteins (i.e., INK4 family), including p21/WAF-1 [56].

Visconti et al. [57] suggested a new role of VDR in the maintenance of the homeostasis of the skin barrier. Altered localization and formation of tissue junction proteins (that are crucial for the formation and maintenance of the paracellular barrier and for cell polarity in simple epithelia and endothelia) in the epidermis have been described in plaque-type psoriasis. An immunohistochemical study investigated the expression of VDR and tight junctions (TJ) proteins (claudin 1, ZO-1, and occludin) in psoriatic skin (n = 20). A reduction of VDR, claudin-1, and ZO-1 expression in psoriatic skin if compared with normal skin was observed, and the statistical analysis showed a significant correlation between a downgrading of VDR expression and that of claudin-1 ( $P < .005$ ) and ZO-1 ( $P < .005$ ). It was concluded that VDR status appears to be associated with the expression level and functions of TJ proteins, suggesting multiple and different cellular functions of the VDR.

Data analyzing VDR expression and genotype in psoriasis are somewhat conflicting [58–67], some studies report a correlation between VDR expression and individual VDR genotypes and the skin eruptions of psoriasis, as well as with responsiveness to treatment with vitamin D analogs. Although no differences in VDR genotype between controls and psoriasis patients were reported at the BsmI site, some studies reported significant difference in terms of ApaI SNP [60] and FokI SNP [61]. Additionally, it has been reported that VDR genotypes are not associated with clinical response to calcipotriol (a topical analog of 1,25(OH)<sub>2</sub>D<sub>3</sub> used in psoriasis) at least in Korean psoriasis patients [62]. Kontula et al. [63] and Mee et al. [64] studied the BsmI polymorphism and the response to calcipotriol treatment in psoriatic patients and found no association between them. According to Colin et al. [65], the FokI polymorphism was associated with the response to calcipotriol, and under conditions of vitamin D insufficiency, this finding might have clinical implications.

Richetta et al. [66] genotyped 108 patients with psoriasis and 268 healthy controls at 5 VDR polymorphisms (A-1012G, FokI, BsmI, ApaI, and TaqI) by TaqMan allelic-discrimination real-time polymerase chain reaction and found a significant increased overall risk of psoriasis for the VDR A-1012G promoter polymorphism (odds ratio [OR] = 2.43, 95% confidence interval [CI]: 1.15–5.13;  $P = .05$ ). A significant higher frequency ( $P = .035$ ) of the A allele was found in psoriatic cases compared with controls. In a case–case analysis, a

statistically significant association between A-1012G and family history emerged ( $P = .033$ ). Furthermore, a significant association of A-1012G risk genotypes with a lower expression of VDR mRNA emerged ( $P = .0028$ ). Results of this study indicated that VDR promoter A-1012G polymorphism is associated with psoriasis risk and suggest that this polymorphism may modulate psoriasis risk by affecting VDR expression.

A recent metaanalysis [67] that included a total of 16 studies with 2086 patients and 2182 controls on VDR polymorphisms and psoriasis indicated an association between psoriasis and the VDR TaqI TT genotype in Caucasian (OR = 1.29, 95% CI = 1.00–1.66,  $P < .05$ ), but not in Asian (OR = 1.32, 95% CI = 0.89–1.96,  $P = .16$ ) populations. However, no association was found between psoriasis and the VDR TaqI polymorphism using dominant, allele contrast or homozygous contrast models. Moreover, that study found no association between psoriasis and either the VDR ApaI, BsmI, or FokI polymorphisms by metaanalyses of the allele contrast, recessive, or dominant models or homozygous contrast models in the overall, Caucasian or Asian populations. The authors of that study concluded that polymorphisms in VDR ApaI, BsmI, and FokI are not associated with psoriasis susceptibility in overall, Caucasian or Asian populations. However, this study provides convincing evidence that VDR TaqI polymorphism is associated with psoriasis susceptibility in Caucasian populations.

As shown before, many association studies between psoriasis and VDR gene have been conducted to date, but the results are controversial. Moreover, the clinical response to the antipsoriatic activity of vitamin D<sub>3</sub> analogs has been reported to be variable. To solve these controversies, a study was conducted recently to explore whether VDR gene polymorphisms are associated with psoriasis susceptibility and clinical response to calcipotriol in psoriatic patients [68]. A total of 110 patients and 183 controls were genotyped for VDR gene polymorphisms rs2228570, rs731236, rs1544410, and rs7975232 by LDR method. SNP-based and haplotype-based association analyses were subsequently performed. Patients with PASI < 3 were treated with calcipotriol ointment monotherapy. After 6 weeks of therapy, the correlations between efficacy and the genotypes of each VDR polymorphism were evaluated. The results for rs7975232 demonstrated that allele A was significantly overrepresented in patients with psoriasis as compared with healthy controls (39.09% vs. 27.05%, OR (95% CI) = 1.731 (1.213–2.471)). Compared with the reference CC genotype, the following ORs were observed: AA genotype OR = 2.404 (95% CI: 1.085–5.328;  $P = .034$ ) and GA genotype OR = 2.143 (95% CI: 1.283–3.579;  $P = .005$ ). Haplotype analyses showed that the rs2228570/rs731236/rs1544410/



rs7975232 CTGA was significantly overrepresented in psoriasis patients compared with controls (OR (95% CI) = 1.907 (1.132–3.214);  $P = .020$ ). Analyzing patients with PASI < 3, the response rates to calcipotriol were significantly higher in patients with rs7975232 CC genotypes than in those with other genotypes ( $\chi^2 = 9.172$ ,  $P = .010$ ). In conclusion, results of this study suggest that VDR polymorphisms are associated with psoriasis susceptibility and clinical response to calcipotriol in psoriatic patients.

Data concerning serum levels of  $1,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}$  in psoriatic patients are conflicting. Some studies report reduced levels of  $1,25(\text{OH})_2\text{D}_3$  in patients with manifest disease [69]. The interplay between vitamin D metabolites and the immunologic milieu in psoriatic skin has become of increasing interest. Psoriasis has been described as a prototypic Th17-mediated disease, and several reports highlight the relevance of an interplay between Th17 and  $1,25(\text{OH})_2\text{D}_3$  for the pathogenesis of this disease. Both factors appear to be entangled in analogous immunologic pathways, with IL-17 being proacanthotic, proinflammatory, and proangiogenic. Moreover, both appear to exert important opposing roles in innate and adaptive immunity, the interaction between which is considered to be of high relevance for the pathogenesis of psoriasis. Additionally,  $1,25(\text{OH})_2\text{D}_3$  has been shown to inhibit Th17 cell function, thereby suppressing its downstream cytokines, including IL-17. On the other hand, the reported decrease in total serum  $25(\text{OH})\text{D}$  levels has been suggested to be a result of the inflammatory milieu created by IL-17. The mean serum IL-17 was significantly higher ( $10.54 \pm 0.38$  pg/mL), and serum total  $25(\text{OH})\text{D}$  was significantly lower ( $21.05 \pm 3.66$  ng/mL) in psoriasis patients ( $n = 48$ ) than in age-, sex-, skin phototype-, and socioeconomic-matched controls ( $n = 40$ ) ( $P = .000$ ). No significant correlations were detected between IL-17 and serum  $25(\text{OH})\text{D}$  concentrations, or between IL-17 and  $25(\text{OH})\text{D}$  and any of the demographic or clinical data collected. These findings indicate a possible role played by Th17 cells and vitamin D insufficiency in the complex pathogenesis of psoriasis; however, their intertwined relationship has to be verified in future investigations [70]. Additionally, the coincidence of pustular psoriasis with hypocalcemia [71] and the exacerbation of psoriasis under chloroquine therapy (thereby reducing total  $1,25(\text{OH})_2\text{D}_3$  levels via inhibition of CYP27B1) are well known [72].

$1,25(\text{OH})_2\text{D}_3$  may also inhibit keratinocyte proliferation at least in part by upregulating leukocyte elastase inhibitor (serpin B1).  $1,25(\text{OH})_2\text{D}_3$ -treated and untreated HaCaT cells (multinucleated transformed keratinocytes) were separated by 2-D differential gel electrophoresis (2DE). Then, the 2DE profiles were analyzed to screen for differentially expressed proteins, which were

identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The upregulation of serpin B1 was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot analysis. The effect of serpin B1 on HaCaT proliferation was analyzed by RNA interference experiments, methylthiazolotetrazolium assay, and flow cytometry. Reproducible 2-DE profiles of HaCaT were established. It was observed that serpin B1 was upregulated in the  $1,25(\text{OH})_2\text{D}_3$ -treated cells that were confirmed by qRT-PCR and Western blot analysis.  $1,25(\text{OH})_2\text{D}_3$  inhibited proliferation of HaCaT cells at concentrations of  $10^{-9}$ – $10^{-6}$  mol/L. HaCaT cell proliferation was promoted when serpin B1 activity was inhibited. Serpin B1 was overexpressed in  $1,25(\text{OH})_2\text{D}_3$ -treated HaCaT cells and may play an important role in inhibiting HaCaT proliferation [73].

The synthetic  $1,25(\text{OH})_2\text{D}_3$  analog calcipotriol increases hCAP18 mRNA expression but inhibits extracellular LL37 peptide production in IL-17/IL-22-stimulated normal human epidermal keratinocytes [74]. Cathelicidin (LL37) serves as not only antimicrobial peptide but also as autoinflammatory mediator. Analogs, such as calcipotriol, are used as topical treatment for psoriasis. However, the effect of calcipotriol on the mRNA expression/production of human cathelicidin antimicrobial protein (hCAP18) and LL37 peptide by IL-17A/IL-22-stimulated keratinocytes remains controversial. To evaluate the modulatory action of calcipotriol on the production of hCAP18 and LL37, hCAP18 mRNA expression was analyzed as well as hCAP18/LL37 peptide production in IL-17A/IL-22-stimulated cultured human keratinocytes by real-time qPCR, ELISA, Western blotting, and immunocytochemistry. By Western blotting, hCAP18 protein was detected in keratinocytes cultured for 72 hours with IL-17/IL-22. Calcipotriol increased hCAP18 mRNA expression in IL-17/IL-22-stimulated keratinocytes. However, LL37 peptide in the culture supernatants was reduced by calcipotriol. Immunostaining revealed that the overproduced LL37 resides within the cells. LL37 promotes psoriasis via interaction with extracellular DNA but may suppress psoriasis by interfering with cytosolic DNA.

## 5. Clinical use of $1,25(\text{OH})_2\text{D}_3$ and its analogs in psoriasis and other skin diseases

The use of  $1,25(\text{OH})_2\text{D}_3$  and its analogs for the treatment of psoriasis resulted from two independent lines of investigation. Because psoriasis is a hyperproliferative skin disorder, it seemed reasonable that the antiproliferative effects of  $1,25(\text{OH})_2\text{D}_3$  could be used for the treatment of this disease. Although it was known that  $1,25(\text{OH})_2\text{D}_3$  was extremely potent in inhibiting

keratinocytes proliferation before launching clinical trials in 1985, MacLaughlin and associates reported the observation that psoriatic fibroblasts were partially resistant to the antiproliferative effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> [75]. This observation prompted MacLaughlin and associates to speculate that 1,25(OH)<sub>2</sub>D<sub>3</sub> may be effective in the treatment of the hyperproliferative skin disease psoriasis if pharmacologic doses were employed. The other line of investigation resulted from a clinical observation. In 1985, Morimoto and Kumahara reported that a patient, who was treated orally with 1 $\alpha$ (OH)D<sub>3</sub> for osteoporosis, had a dramatic remission of psoriatic skin lesions [76]. Morimoto et al. reported a follow-up study, demonstrating that almost 80% of 17 patients with psoriasis who were treated orally with 1 $\alpha$ (OH)D<sub>3</sub> at a dose of 1.0  $\mu$ g/day for up to 6 months showed clinically significant improvement [77].

Currently, numerous studies have reported that various vitamin D analogs, including calcitriol, calcipotriol, tacalcitol, hexafluoro-1,25(OH)<sub>2</sub>D<sub>3</sub> [78], and maxacalcitol, are effective and safe in the topical treatment of psoriasis [79–87]. It has been shown that topical calcitriol and its analogs are very effective and safe for the long-term treatment of psoriasis [84–86]. Applied twice daily topically in amounts of up to 100 g of ointment (50  $\mu$ g calcipotriol/g ointment) per week, calcipotriol was shown to be slightly more effective in the topical treatment of psoriasis than betamethasone 17-valerate ointment [86]. Efficacy of topical treatment with maxacalcitol was compared with topical calcipotriol treatment [82]. In this study, investigators' overall assessment suggests that maxacalcitol 25  $\mu$ g/g may be more effective than once-daily calcipotriol (50  $\mu$ g/g). It has been reported that a mild dermatitis can be seen in about 10% of patients treated with calcipotriol (50  $\mu$ g/g), particularly on the face [87]. This side effect (mild dermatitis on the face) is not reported after topical treatment with calcitriol. Allergic contact dermatitis to vitamin D analogs is very rare; however, cases with allergic contact dermatitis to other ingredients of the ointment including propylene glycol have been reported [88–90]. The most common adverse event observed in psoriasis patients treated with maxacalcitol (6–50  $\mu$ g/g maxacalcitol ointment) was a burning sensation of the target plaque [82]. In three out of four patients developing this side effect in one study, symptoms were severe enough to require discontinuation of the treatment [82].

A double-blind, right/left comparison, placebo-controlled evaluation demonstrated efficacy and safety of topical treatment with hexafluoro-1,25(OH)<sub>2</sub>D<sub>3</sub> (5  $\mu$ g/g) in psoriasis patients [83]. Adverse events included mild irritation. This irritation did not necessitate discontinuation of the study medication. During the large area topical administration study period, a

cobblestone appearance was initially noted in a few patients. This resolved with continued therapy after 3–4 weeks. Hexafluoro-1,25(OH)<sub>2</sub>D<sub>3</sub>-treated plaques also developed very mild perilesional scales as observed with other vitamin D analogs [83]. Efficacy and safety of topical treatment with tacalcitol (4 and 20  $\mu$ g/g) has been shown as well [83–85,91]. In one study, tacalcitol treatment was generally well tolerated, and there were no serious or unexpected adverse events reported. However, discontinuation of treatment as a result of skin irritation was seen in 5.9% of these patients [85]. The greatest frequency of cutaneous side effects occurred during initial treatment, and the incidence decreased markedly as the treatment was well tolerated with continued use [85].

The results of four separate studies designed to evaluate specific local-safety parameters of various vitamin D analogs including cumulative irritancy, cutaneous contact sensitization, photoallergic contact sensitization, and phototoxicity, were analyzed [92]. 1,25(OH)<sub>2</sub>D<sub>3</sub> (3  $\mu$ g ointment) was classified as nonirritant when compared with calcipotriol, tacalcitol, and white petrolatum (control). Petrolatum and tacalcitol were slightly irritant and calcipotriol moderately irritant. No sensitization was observed with 1,25(OH)<sub>2</sub>D<sub>3</sub> (3  $\mu$ g ointment). Regarding phototoxic potential, sites treated with calcitriol 3  $\mu$ g ointment or vehicle ointment were less irritated than those treated with white petrolatum or those that were untreated. Using standard photoallergenicity testing methodology, there were no skin reactions of a photoallergic nature to the study material [92].

A long-term follow-up study demonstrated the efficacy and safety of oral calcitriol as a potential treatment of psoriasis [93]. Of the 85 patients included in that study that received oral calcitriol for 36 months, 88.0% had some improvement in their disease, whereas 26.5%, 26.3%, and 25.3% had complete, moderate, and slight improvement in their disease, respectively. Serum calcium concentrations and 24 hours urinary calcium excretion increased by 3.9% and 148.2%, respectively, but were not outside the normal range. Bone mineral density of these patients remained unchanged. A very important consideration for the use of orally administered calcitriol is the dosing technique. To avoid its effects on enhancing dietary calcium absorption, it is very important to provide 1,25(OH)<sub>2</sub>D<sub>3</sub> at night time. Perez et al. [93] showed that as a result of this dosing technique, doses of 2–4  $\mu$ g/night 1,25(OH)<sub>2</sub>D<sub>3</sub> are well tolerated by psoriatic patients.

In the 1930s, very high doses of vitamin D were found to be very effective in treating psoriasis [1–3]. However, due to severe toxicity, this practice was halted. Coimbra and colleagues resurrected high-dose vitamin D therapy and avoided severe hypercalcemia by placing their patients on a zero-calcium diet [94]. They reported 9

patients with extensive plaque psoriasis on 35,000 IUs of vitamin D<sub>3</sub> daily for 6 months. These patients were on a strict low-calcium diet avoiding dairy products and calcium-enriched foods with good hydration of at least 2.5 L of water daily. They demonstrated dramatic improvement in the Psoriasis Area and Severity Index (PASI) score for all 9 patients. They reported that the mean baseline 25(OH)D was  $18.4 \pm 8.9$  ng/mL, and at the end of the trial the concentration was  $106.3 \pm 31.9$  ng/mL. They reported that the serum concentrations of PTH significantly decreased from  $57.8 \pm 16.7$  to  $28.9 \pm 8.2$  pg/mL, and there was no significant change in the serum calcium concentrations ( $9.7 \pm 0.7$  baseline and 6 months later  $9.4 \pm 0.7$  mg/dL) [94]. Recently, Mahtani et al. [95] using the Coimbra protocol reported 6 cases of psoriasis treated with daily oral vitamin D<sub>3</sub> in doses ranging from 30,000 IU to 60,000 IU over a period of 2–6 months and then followed by lower daily maintenance doses were reported. The dose of vitamin D<sub>3</sub> was adjusted based on the drop in the level of parathyroid hormone as the ionized calcium levels were also periodically monitored to prevent hypercalcemia. The rationale for this treatment with relatively high doses of vitamin D was the growing body of evidence published in the scientific literature suggesting that vitamin D<sub>3</sub> resistance caused by polymorphisms in genes that contribute to vitamin D metabolism may have a potential role in the pathoprogenesis of psoriasis. Notably, complete control of psoriasis was observed within a span of 2–6 months, which was assessed by PASI and a symptom Visual analog scale. The authors concluded that supervised, daily oral higher than usual vitamin D<sub>3</sub> can be given safely as an effective therapeutic modality for treating psoriasis [95].

Patients with psoriasis may need intermittent treatment for their entire lives. Vitamin D analogs have been shown not to exhibit tachyphylaxis during treatment of psoriatic lesions, and topical treatment can be continued indefinitely.

### 5.1 Treatment of scalp psoriasis

A double-blind, randomized multicenter study demonstrated that calcipotriol solution is effective in the topical treatment of scalp psoriasis [96–98]. 49 patients were treated twice daily over a 4-week period [96]. 60% of patients on calcipotriol showed clearance or marked improvement versus 17% in the placebo group. No side effects were reported.

### 5.2 Treatment of nail psoriasis

The occurrence of nail psoriasis has been reported in up to 50% of patients. Nails, in general, are very difficult

to treat and respond slowly. Until now, there has been no consistently effective treatment for psoriatic nails. It has been reported that calcipotriol ointment is effective in the treatment of nail psoriasis [99].

### 5.3 Treatment of face and flexures

Although the use of calcipotriol ointment is not recommended on face and flexures because of irritancy, most patients tolerate vitamin D analogs on these sites. It has been shown that calcitriol ointment (3 µg of 1,25(OH)<sub>2</sub>D<sub>3</sub> per gram of petrolatum) was found to be better tolerated and would appear to be more effective than calcipotriol ointment (50 µg of calcipotriol per gram of petrolatum) in the treatment of psoriasis in sensitive areas [100].

### 5.4 Treatment of skin lesions in children

During the past few years, it has been shown that topical application of vitamin D analogs including calcitriol ointment (3 µg of 1,25(OH)<sub>2</sub>D<sub>3</sub> per gram of petrolatum) is an effective, safe, and reliable therapy to cure psoriatic skin lesions in children [101–103].

### 5.5 Treatment of psoriatic lesions in HIV patients

We have treated an HIV-positive patient suffering from psoriatic skin lesions with topical and oral 1,25(OH)<sub>2</sub>D<sub>3</sub>. The patient responded well, and there was no evidence of enhancement in HIV-disease activity or alterations in the number of T lymphocytes, or CD4<sup>+</sup>, and CD8<sup>+</sup> cells.

### 5.6 Combination of vitamin D analogs with other therapies

It has been reported that efficacy of topical treatment with vitamin D analogs in psoriasis can be increased by combination with other therapies, including methotrexate (MTX), very-low-dose oral cyclosporine (2 mg/kg/day), oral acitretin, topical dithranol, topical steroids, PUVA (psoralen plus UV-A) and UV-B, or narrow band UV-B phototherapy [104–112]. It has been shown that the combination of calcipotriol and MTX is safe and well tolerated [111]. The combination resulted in lower cumulative dosages of MTX compared with MTX and vehicle. Therefore, the risk of MTX-induced side effects is substantially decreased [111]. Addition of calcipotriol ointment to oral application of acitretin (a vitamin A analog) was shown to produce a significantly better treatment response achieved with a lower cumulative dose of acitretin in patients with severe

extensive psoriasis vulgaris, as compared with the group of patients treated with oral acitretin alone. The number of patients reporting adverse events was similar between the two treatment groups [106].

Complete clearing or 90% improvement in PASI was observed in 50% of patients treated with calcipotriol/cyclosporine versus 11.8% in the placebo/cyclosporine group. No difference was found in that study between the groups in short-time side effects. Kragballe and co-workers reported that efficacy of topical calcipotriol treatment in psoriasis can be improved by simultaneous UV-B phototherapy. Combination therapy of psoriasis with topical calcipotriol and narrow-band UV-B has been shown to be very effective for the treatment of psoriatic plaques [108]. One can speculate whether the therapeutic efficacy of UV-B in psoriasis may be at least in part because of increased cutaneous vitamin D synthesis. It has been shown that the combination of topical treatment with vitamin D analogs and UV-radiation does not alter the tolerability or safety of therapy [113]. Vitamin D analogs may be topically applied at any time up to 2 hours before or immediately after UV radiation [102]. Results of a controlled, right/left study have demonstrated that pretreatment of psoriasis with the vitamin D<sub>3</sub> derivative tacalcitol increases the responsiveness to 311-nm UV-B [114]. Additionally, it was shown that tacalcitol ointment (4 µg/g) and 0.1% tazarotene gel are both comparably effective in improving the therapeutic result of PUVA therapy in patients with chronic plaque-type psoriasis [115]. The treatment requirements to induce complete or near complete clearing were significantly lower for both combination treatments than for PUVA monotherapy ( $P < .01$ ). The median cumulative UV-A dose and number of exposures were 30.6 J/cm<sup>2</sup> (95% CI 22.5–71.2) and 14 (95% CI 11–16) for tacalcitol plus PUVA, 32.3 J/cm<sup>2</sup> (95% CI 22.5–73.8) and 14 (95% CI 11–19) for tazarotene plus PUVA, and 37.0 J/cm<sup>2</sup> (95% CI 29.5–83.9) and 16 (95% CI 14–22) for PUVA monotherapy. No difference between the three regimens was observed with regard to duration of remission. Adverse reactions occurred more often with 0.1% tazarotene than with tacalcitol but were, in general, mild and completely reversible on using a lower concentration of 0.05% tazarotene. It has been concluded that besides accelerating the treatment response, both agents, by virtue of their UV-A dose-sparing effect, might also help to reduce possible long-term hazards of PUVA treatment. Previously, a case report described two patients treated with a combination treatment of calcipotriol and bath psoralens and UV-A who developed hyperpigmentation at the lesional sites where calcipotriol ointment was applied [116].

Combined topical treatment with calcipotriol ointment (50 µg/g) and betamethasone ointment was shown to be slightly more effective and caused less skin irritation than calcipotriol used twice daily [107]. A vehicle has been created with the objective of obtaining optimal stability of both calcipotriene and betamethasone dipropionate in the combination product. Early onset of action and efficacy of a fixed combination of calcipotriene and betamethasone dipropionate in this vehicle in the treatment of psoriasis has been reported, making it a standard therapy for the topical treatment of psoriasis [117].

Augustin et al. [118] evaluated scientific evidence about topical long-term therapy with 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs, corticosteroids, and their two-compound products in psoriasis vulgaris and scalp psoriasis and developed daily practice recommendations. Best evidence regarding topical long-term treatment was available for the two-compound formulation containing calcipotriene and betamethasone. In a comparative trial in psoriasis vulgaris, the two-compound formulation showed superior tolerability and cost-effectiveness compared with monotherapy. In scalp psoriasis, the two-compound gel was superior compared with calcipotriene monotherapy. The authors concluded that because of a favorable risk–benefit ratio in maintenance trials and better cost-effectiveness, the application of two-compound products once or twice a week after initial therapy is recommended. It has been hypothesized that calcipotriol counteracts glucocorticoid-induced skin atrophy, which is associated with changes in the ECM. To elucidate the combined effects of calcipotriol and betamethasone on key ECM components, a comparative study to the respective monotreatments was carried out. The effect on collagen I synthesis, matrix metalloproteinase (MMP) secretion, and hyaluronic acid (HA) production was investigated in primary human fibroblast and keratinocyte cultures as well as in a human skin explant model. In that study, calcipotriol counteracted betamethasone-induced suppression of collagen I synthesis. Similarly, calcipotriol and betamethasone had opposing effects on MMP expression in both fibroblasts and keratinocytes. Moreover, calcipotriol was able to restore betamethasone-impaired HA synthesis in keratinocytes and prevented betamethasone-induced epidermal thinning in minipigs on treatment with a calcipotriol/betamethasone gel. In summary, these results showed for the first time in primary human skin cultures that calcipotriol reduces early signs of betamethasone-induced skin atrophy by modulation of key ECM components. These results indicate that the calcipotriol component of the fixed combination gel counteracts the atrophogenic effects of betamethasone on the skin [119].



## 6. Vitamin D in prevention and therapy of other skin diseases

### 6.1 Ichthyosis

A double-blind, bilaterally paired, comparative study has demonstrated the effectiveness of topical treatment with calcipotriol ointment on congenital ichthyoses [120]. Reduction in scaling and roughness on the calcipotriol-treated side was seen in all patients with lamellar ichthyosis and bullous ichthyotic erythroderma of Brocq. The only patient treated with Comel-Netherton syndrome showed mild improvement, whereas the only patient suffering from ichthyosis bullosa of Siemens who was treated with calcipotriol did not show any change in severity on the calcipotriol-treated as compared with the vehicle-treated side. It has been reported that topical tacalcitol therapy was ineffective against ichthyoses that are characterized by retentive hyperkeratosis and a lack of epidermal hyperproliferation, including X-linked ichthyosis, ichthyosis vulgaris, and acquired ichthyosis [121].

Although the use of various active vitamin D analogs for treating various forms of ichthyosis has proven to be somewhat effective, short-term high-dose vitamin D in children with congenital ichthyosis was reported to be very effective. Seven children with congenital ichthyosis (five with the autosomal recessive congenital ichthyosis; two with epidermolytic ichthyosis) who were severely vitamin D deficient and had rickets were given 60,000 IUs vitamin D<sub>3</sub> daily for 10 days. The children were then placed on 400–600 IUs daily. The authors reported significant improvement in scaling by day 5, showing further improvement by day 10 in 6 of the 7 cases. At 1 month, the skin had become nearly normal in all cases of autosomal recessive congenital ichthyosis. Significant reduction in stiffness was also observed in all of the children [122].

### 6.2 Scleroderma

Preliminary findings point to the efficacy of vitamin D analogs for the treatment of scleroderma. Humbert et al. [123] reported that oral administration of 1.0–2.5 µg/day calcitriol improves skin involvement, probably via inhibition of fibroblast proliferation and dermal collagen deposition. Notably, it was recently demonstrated that several vitamin D hydroxyderivatives, including noncalcemic 20(OH)D<sub>3</sub>, generated by CYP11A1 action on vitamin D<sub>3</sub>, exert antifibrotic activity in human dermal fibroblasts and in a bleomycin-based mouse model of scleroderma [124]. In that study, it was shown that the antiproliferative and antifibrotic activities of these vitamin D hydroxyderivatives depend on the functional integrity of retinoic orphan

receptor (RORγ). In that study, all vitamin D analogs tested inhibited TGF-β1-induced collagen synthesis in RORγ+/+ fibroblasts and the expression of other fibrosis-related genes. Notably, this effect was curtailed or reversed in RORγ–/– fibroblasts. Treatment with 20(OH)D<sub>3</sub> or 1,20(OH)<sub>2</sub>D<sub>3</sub> exerted strong changes in the transcriptomes of fibroblasts of RORγ–/– versus wild-type mice were seen. The authors concluded that these findings provide a molecular basis to explain, at least in part, the observed phenotypic differences [124].

### 6.3 Vitamin D and autoimmune bullous diseases of the skin

Many epidemiological studies have linked vitamin D deficiency to an increased risk for the development of various autoimmune diseases, including diabetes mellitus type 1, rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, or systemic lupus erythematosus. More recently, such a connection was also proposed for several skin autoimmune diseases, including autoimmune bullous diseases (AIBD). AIBD represents a group of relatively rare and potentially life-threatening chronic inflammatory skin diseases, which can be categorized into three different subgroups, namely pemphigus vulgaris (PV), bullous pemphigoid (BP), and dermatitis herpetiformis (DH), which are all characterized by the presence of many different tissue-bound and circulating autoantibodies. Dermatitis herpetiformis represents the cutaneous manifestation of celiac disease with autoantibodies directed against the tissue and the epidermal transglutaminase [125]. The term pemphigus comprises a group of autoantibody-mediated autoimmune diseases in which the loss of cell adhesion (acantholysis) can cause blisters and erosions in the skin and mucosa. There are two major subtypes of pemphigus diseases, its most common form pemphigus vulgaris (PV) and pemphigus foliaceus (PF), characterized by the presence of autoantibodies directed against cell–cell adhesion molecules found in desmosomes in the epidermis, namely desmoglein (Dsg) 1 and desmoglein 3 [126]. Bullous pemphigoid (BP), characterized by autoantibodies to BP180 (collagen type XVII or COL17) and BP230, is the most common autoimmune subepidermal blistering disorder, whose incidence, similarly to other autoimmune diseases, is constantly increasing [125]. By contrast, epidermolysis bullosa acquisita (EBA), a subepidermal blistering disease with autoantibodies to type VII collagen (COL7), is one of the rarest AIBDs, with an incidence rate of 0.2–0.5 per million per year [127].

While the etiology of pemphigus diseases and BP is mostly unknown, several genetic and environmental

risk factors have been proposed. PV, PF, and BP all are frequently associated with other (auto) inflammatory diseases, such as psoriasis, neurological and psychiatric disorders, or some malignancies. In the case of PV, genetic risk factors include either HLA alleles DRB1\*04:02 and DQB1\*05:03 or non-HLA genes, i.e., DSG3, TAP2, IL6, and ST18. In addition, an association between HLA-DQB1\*0301 and BP or mucous membrane pemphigoid (MMPem), as well as the risk allele HLA-DRB1\*15:03 or association between HLA-DR2 and EBA, were reported [128,129]. In PV, environmental risk factors, in particular use of penicillamine and captopril, as well as exposure to pesticides, metal vapor, UV, ionizing radiation, burns, undergoing surgery or stressful life events, have been noted. For BP, several environmental or genetic risk factors, including trigger factors, such as penicillin, vancomycin, gentamycin, trauma, burns, radiotherapy, UV radiation, vaccination, and contact allergy to metals, have been described [127,128]. The majority of studies investigating the pathophysiology of pemphigoid are based on experimental animal models and numerous evidence pointed to pathogenic importance of both the local and systemic innate and adaptive autoimmune responses against structural proteins of the dermal–epidermal junction [128,129].

In genetically susceptible individuals, the autoimmune reaction is driven by autoreactive T and B lymphocytes. Autoreactive T cells are educated by APCs that present Dsg peptides via HLA class II molecules. Consequently, autoreactive T helper cells specific for Dsg molecules drive generation of autoreactive B cells and secretion of the tissue-bound and circulating autoantibodies to Dsg [126,130].

Pemphigus can be treated with systemic corticosteroids and adjuvant therapies, including immunosuppressive agents, intravenous immunoglobulin (IVIG), and plasmapheresis. In addition, rituximab, a monoclonal antibody against the CD20 molecule (B cells' marker), is another promising therapeutic option [126]. As in the case of PV, BP can be controlled medically by using corticosteroids, high doses of IVIG, rituximab, plasmapheresis, and immunoadsorption [128,131].

### 6.3.1 Vitamin D status in autoimmune bullous diseases

In recent years, the role of vitamin D has been investigated in AIBD [29,40,132–143], and emerging evidence suggests an increased frequency of vitamin D deficiency/insufficiency in patients with pemphigus and pemphigoid, such as the PV and BP or EBA, respectively. In addition, in some reports, lower concentrations of 25(OH)D have been associated with AIBD activity, pointing toward a possible causative role of hypovitaminosis D in the disease process [132,133,142]. However, some other studies have found no difference in the

25(OH)D levels between patients and healthy subjects, possibly due to limited number of patients and controls involved in these studies [136,140,143].

### 6.3.2 Experimental therapies using vitamin D analogs in autoimmune bullous diseases

While patients with the autoimmune bullous skin diseases suffer from vitamin D deficiency [29,40,132–143], a causal relationship between hypovitaminosis D and the development of autoimmune bullous diseases has not been proven. It is hypothesized that binding of BP-specific IgG autoantibodies to BP180 initiates the Fc receptor-independent events, leading to the excessive expression and secretion of proinflammatory IL-6 and IL-8 from basal keratinocytes [128,144]. Activation of complement at the dermal–epidermal junction (DEJ), together with the mast cells' degranulation and inflammatory chemokines, results in the infiltration of inflammatory cells, including granulocytes, in the upper dermis. The reactive oxygen species (ROS) and matrix metalloproteinases (MMP) released by the activated granulocytes induce dermal–epidermal splitting and blister formation [128]. It was reported that BP IgG-induced IL-6 and IL-8 secretion from human keratinocytes HaCaT cells was reduced in the presence of calcitriol via inhibition of STAT3 phosphorylation and NF- $\kappa$ B activity [40]. Effectiveness of the calcitriol treatment was confirmed in vivo by using a well-established EBA mouse model, which offers an elegant tool to study the pathogenesis of autoantibody-induced and immune cell-mediated blistering, as well as enables investigators to identify new therapeutic targets for EBA and other AIBD [129,145]. It was found that orally administrated calcitriol modulated the clinical course of experimental EBA through induction of the T and B regulatory cells, as well as downregulation of neutrophil activity and blockade of proinflammatory Th17 cell population [29]. Because both, the immunosuppressive Treg and Breg, or proinflammatory Th1, Th17, and neutrophils are associated with the AIBD development [29,146–150], targeting these cell populations is an important therapeutic approach in AIBD.

In conclusion, despite growing understanding of AIBD pathogenesis, treatment of this group of rare skin disorders remains challenging. This is because of frequent relapses, numerous side effects due to corticosteroid usage, or simply due to lack of effective treatment [131,151]. In addition, the incidence of AIBD is constantly increasing [125], and therefore, there is a growing urgency for discovering an effective treatment or prophylactic regimen to reduce the incidence of these autoimmune disorders. Despite the usage of topical vitamin D analogs in the treatment of autoimmune skin conditions, such as psoriasis and vitiligo [141,152], there are only a limited number of

epidemiological and experimental studies on vitamin D involvement in autoimmune bullous diseases. This emerging topic requires further research including clinical trials investigating the effect of topically or systemically applied pemphigus and pemphigoid patients. In fact, several case reports have described the efficacy of either orally or topically applied vitamin D analogs [135,138,153] in Hailey–Hailey disease, also known as a familial benign chronic pemphigus. Finally, because there are many case reports describing the coexistence of AIBD and psoriasis [154], the role of topically applied vitamin D analogs in the treatment of these disorders needs to be properly evaluated.

#### 6.4 Vitamin D and hair diseases

Hair diseases, presenting with scarring or nonscarring hair loss, are a common problem that affects both male and female patients [155,156]. Since any disturbances in the cycling of hair follicles may lead to hair shedding, or alopecia, and considering the pluripotent biologic effects of vitamin D compounds, it is not surprising that the relevance of vitamin D for hair diseases was investigated in many studies. The role of VDR has been well established and extensively studied in the hair cycle. Its deficiency is also closely linked to several types of alopecia, including alopecia areata, telogen effluvium, and androgenetic alopecia (AGA). The scientific rationale why vitamin D derivatives may represent promising candidates to prevent and to treat hair disorders and diseases is based on the ability of these compounds to regulate many cellular mechanisms that are of importance for hair growth, including their anti-inflammatory and immunomodulatory properties and their ability to regulate keratinocyte differentiation and proliferation. Moreover, the expression of the VDR was shown in key structures of the hair follicle, making them to targets of vitamin D activity. In line with these findings, it was shown that vitamin D hydroxyderivatives regulate the hair cycle, and their role in hair loss is under constant research. A recent systematic review summarized our present scientific knowledge how the vitamin D endocrine system regulates many of the various signaling pathways that affect growth and differentiation of hair follicles. Notably, vitamin D deficiency was in that study also associated with scarring alopecia.

Most studies included into that review showed an inverse relationship between serum 25(OH)D levels in patients with different types of nonscarring alopecia, which could suggest a contribution of the vitamin D endocrine system to the pathogenesis of hair loss and nonscarring alopecias including telogen effluvium, androgenetic alopecia (AGA), alopecia areata, and

trichotillomania. Gerkowicz et al. [155] review of the literature (searching PubMed and Google Scholar databases) identified 13 relevant articles with a focus on AGA, male pattern baldness, and serum 25(OH)D levels [157]. The authors concluded that serum vitamin D status might be a possible parameter for diagnosing the onset and severity of AGA. They report that vitamin D supplementation has proven to be useful in the regrowth of hair in nonhuman subjects. In their opinion, oral vitamin D supplementation or topical application of vitamin D compounds may therefore represent a valid therapeutic approach in many hair diseases including androgenetic alopecia, where topical calcipotriol has been suggested to be a promising treatment option to regrow hair follicles and prevent miniaturization of follicles.

To clarify the association of alopecia areata (AA) with serum vitamin D status and calcium levels, a systematic review and metaanalysis [158] investigated recently all relevant articles published up to February 2020 in PubMed, Embase, and Cochrane Library databases. Data on 1585 patients with AA and 1114 controls from 16 case–control studies and three cross-sectional studies were included in this pooled metaanalysis that was conducted using the random-effects model because of interstudy heterogeneity (vitamin D level,  $I^2 = 87.90\%$ ; vitamin D deficiency,  $I^2 = 81.10\%$ ; serum calcium level,  $I^2 = 83.80\%$ ). A combined analysis revealed that patients with AA had significantly lower mean serum 25(OH)D levels compared with controls (WMD  $-9.08$ , 95% CI  $-11.65$ ,  $-6.50$ ,  $P < .001$ ) and were more likely to have vitamin D deficiency (OR 4.14, 95% CI 2.34, 7.35,  $P < .001$ ). However, the pooled analysis revealed that patients with AA did not have significantly lower serum calcium levels compared with controls (WMD  $-0.17$ , 95% CI  $-0.40$ ,  $0.06$ ,  $P = .143$ ). The authors concluded that screening for vitamin D deficiency and vitamin D supplementation may be beneficial in the treatment of patients with AA. In this context, a recent study in AA compared topical treatment with mometasone 0.1% cream alone to combined treatment with topical calcipotriol 0.005% ointment and mometasone 0.1% cream [159]. In that comparative analytical study with over 100 AA patients, group A patients ( $n = 50$ ) were advised to apply topical mometasone 0.1% cream along with topical calcipotriol 0.005% ointment each once daily, whereas patients of group B ( $n = 50$ ) were advised to apply only topical mometasone 0.1% cream in the same amount, once a day. Follow-up of all patients was done at 6, 12, and 24 weeks, and the outcome was assessed according to the Severity of Alopecia Tool (SALT) score at every visit. Both the groups were statistically comparable in terms of age ( $P = .694$ ) and sex ( $P = .683$ ) distribution. Baseline mean SALT score of group A and group B patients was 7.22 and 6.05,



respectively ( $P = .145$ ). At the end of 24 weeks, mean SALT score of group A and group B patients decreased by 4.24 and 3.39, respectively ( $P < .001$ ). The authors concluded that adding topical calcipotriol 0.005% ointment with topical mometasone 0.1% cream has higher efficacy than topical mometasone alone, in the treatment of alopecia areata.

Recently, a prospective study including 30 AA patients (mean serum 25(OH)D level  $7.65 \pm 4.50$  ng/mL; mean age  $28.9 \pm 9.96$  years; mean SALT score  $35.8 \pm 27.5$ , median disease duration 48 weeks) and 30 healthy controls (mean serum 25(OH)D level  $15.8 \pm 11.47$  ng/mL; mean age  $31.17 \pm 9.43$  years) was performed [160]. Twenty-nine (96.7%) patients were vitamin D deficient (25(OH)D  $< 20$  ng/mL), compared with 22 (73.3%) controls ( $P = .001$ ). Serum 25(OH)D levels inversely correlated with severity of the disease ( $r = -.256$ ,  $P = .17$ , and duration of disease but did not correlate with pattern of AA and VDR expression in tissue samples. VDR expression was reduced in all patients and was normal in controls. Inverse correlation of VDR was noted with presence of inflammation on histology ( $P = .02$ ). VDR upregulation posttreatment was seen only in 13% of patients and demonstrated no correlation with response to treatment. The authors concluded that vitD deficiency in AA correlates inversely with disease severity and duration. VDR expression is reduced in AA and inversely correlates with inflammation histologically but does not correlate with serum 25(OH)D levels, severity, pattern, or duration of illness [160].

## 6.5 Vitamin D and vitiligo

A systematic review and updated metaanalysis, which investigated the vitamin D status in vitiligo patients and which included 31 studies identified by searching PubMed and other databases, was published recently [161]. In this metaanalysis, the random effects model was used to obtain standardized mean differences and pooled correlation coefficients, metaregression and sub-group analyses were conducted to explore heterogeneity, and the presence of publication bias and the study robustness were tested using funnel plot and sensitivity analyses, respectively. Overall, this metaanalysis showed significantly decreased serum vitamin D levels (standardized mean difference =  $-1.03$ ;  $P < .0001$ ) in vitiligo patients compared with controls. Subgroup analysis revealed that vitiligo patients with indoor/urban work had a significantly lower vitamin D level when compared with their outdoor/rural counterparts (standardized mean differences =  $-0.45$ ;  $P = .03$ ). The sensitivity analysis indicated that no single study had a significant influence

on the overall outcome, suggesting the robustness of this metaanalysis. However, this study has some limitations including varied sample sizes and heterogeneous study populations from different countries. Coimbra and colleagues used their same strategy of giving very high doses of vitamin D, 35,000 IUs once daily for 6 months to patients with vitiligo [94]. The 16 patients were instructed to also be on zero calcium diet and to be well hydrated. 14 of the 16 patients with vitiligo had 25%–75% repigmentation. Serum concentrations of total calcium and ionized calcium did not change [94].

## 6.6 Vitamin D and skin aging

It has been shown convincingly by Andrzej Slominski, Georgeta Bocheva [162], and others that distinct cutaneous photoproducts, including active hydroxyderivatives of vitamin D<sub>3</sub> and lumisterol (L<sub>3</sub>) exert a variety of antiaging and photoprotective effects on the skin. These are at least in part achieved through immunomodulation and include beside antiinflammatory actions, regulation of keratinocytes proliferation, and differentiation programs to build and maintain the epidermal barrier necessary for skin homeostasis. In addition, these photoproducts have the capacity to induce antioxidative responses, inhibit DNA damage, and induce DNA repair mechanisms to attenuate premature skin aging and cancerogenesis. The mechanism of action that underlies these antiaging effects involve interaction with multiple nuclear receptors including VDR, AhR, LXR, reverse agonism on ROR $\alpha$  and  $-\gamma$ , and nongenomic actions through 1,25(OH)<sub>2</sub>D<sub>3</sub>-MARRS receptor and interaction with the nongenomic binding site of the VDR. Therefore, it has been concluded that active forms of vitamin D<sub>3</sub> including 1,25(OH)<sub>2</sub>D<sub>3</sub> and nonclassical (CYP11A1-initiated) D<sub>3</sub> derivatives as well as L<sub>3</sub> derivatives are promising agents for the prevention, attenuation, or treatment of premature skin aging. They could be administrated orally and/or topically. Other forms of parenteral application of vitamin D<sub>3</sub> precursor should be considered to avoid its predominant metabolism to 25(OH)D that is not recognized by CYP11A1 enzyme. The efficacy of topically applied vitamin D<sub>3</sub> and L<sub>3</sub> derivatives needs further clinical evaluation in future trials.

## 6.7 Sunscreens and vitamin D status

Global concern about vitamin D deficiency has fueled debates on photoprotection and the importance of solar exposure to meet vitamin D requirements [163]. An international panel of 13 experts in endocrinology, dermatology, photobiology, epidemiology, and biological anthropology reviewed the literature prior to a 1-day



meeting in June 2017, during which the evidence was discussed [163]. Methods of assessment and determining factors of vitamin D status and public health perspectives were examined, and consequences of sun exposure and the effects of photoprotection were assessed. A serum level of  $\geq 50$  nmol/L 25(OH)D is a target for all individuals. Broad-spectrum sunscreens that prevent erythema are unlikely to compromise vitamin D status in healthy populations. Vitamin D screening should be restricted to those at risk of hypovitaminosis, such as patients with photosensitivity disorders, who require rigorous photoprotection. Screening and supplementation are advised for this group. Sunscreen use for daily and recreational photoprotection does not compromise vitamin D synthesis, even when applied under optimal conditions. What's already known about this topic? Knowledge of the relationship between solar exposure behavior, sunscreen use, and vitamin D is important for public health, but there is confusion about optimal vitamin D status and the safest way to achieve this. Practical recommendations on the potential impact of daily and/or recreational sunscreens on vitamin D status are lacking for healthy people. What does this study add? Judicious use of daily broad-spectrum sunscreens with high ultraviolet (UV) A protection will not compromise vitamin D status in healthy people. However, photoprotection strategies for patients with photosensitivity disorders that include high sun-protection factor sunscreens with high UVA protection, along with protective clothing and shade-seeking behavior, are likely to compromise vitamin D status. Screening for vitamin D status and supplementation are recommended in patients with photosensitivity disorders.

## 6.8 Actinic keratoses, skin cancer

It has been reported in the literature that distinct polymorphisms of the VDR gene are associated with the presence of actinic keratoses and nonmelanoma and melanoma skin cancer [164,165], indicating that the vitamin D endocrine system (VDES) may be important for development of cutaneous malignancies. Moreover, some investigations indicate that topically applied vitamin D analogs may be effective in the treatment of actinic keratoses (cutaneous SCC in situ). Although it has been reported that actinic keratoses in renal transplant recipients do not improve with calcipotriol cream and all-trans retinoic acid (RA) cream as monotherapies or in combination during a 6-week treatment period [166]. However, other reports, including an investigator-blinded, half-side comparison trial, indicate that topically applied vitamin D analogs may be effective in the treatment of actinic keratoses [167]. In this

study [167], patients applied calcipotriol cream to one side and Ultrabase cream as placebo to the other side of the scalp and/or face for 12 weeks. The total number of actinic keratoses, diameters, and total scores of the target lesions were determined at each visit. Nine patients were included, eight of whom completed the treatment. There was a statistically significant difference between the total number of actinic keratoses at baseline and at week 12 on the calcipotriol applied side, whereas no difference was detected on placebo-applied side ( $P = .028$  vs.  $P = 1.00$ ). The mean total score of the target lesions reduced significantly at week 12 on calcipotriol side; in contrast, no significant reduction was found on placebo side ( $P = .017$  vs.  $P = .056$ ). In conclusion, although the study was suggestive, the clinical efficacy of vitamin D analogs in the topical treatment of actinic keratoses remains to be elucidated in future investigations.

In vitro studies have demonstrated strong antiproliferative and prodifferentiating effects of vitamin D analogs in many VDR-expressing tumor cell lines, including malignant melanoma, SCC, breast and lung cancer, and leukemic cells [168–172]. In vivo studies supported these results and showed that active-vitamin D analogs block proliferation and tumor progression of epithelial tumors in rats [172]. Inhibition of tumor growth of human malignant melanoma and other cancer xenografts was also demonstrated in immune-suppressed mice but only with high doses of calcitriol [172]. Little is known regarding the effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the formation of metastases in patients with malignant melanoma or SCC of the skin.

## 6.9 Acne

The therapeutic efficacy of vitamin D in acne has been investigated in the middle of the past century [173–176] with limited success. However, more recent laboratory investigations and animal studies indicate that vitamin D compounds may be effective in acne treatment [177]. In the rhino mouse model, a comedolytic effect of topically applied active vitamin D<sub>3</sub> analog maxacalcitol on pseudocomedones was demonstrated [177]. The rhino (*hr<sup>rh</sup>/hr<sup>rh</sup>*) phenotype is because of an autosomal recessive mutation in the hairless (*hr*) gene [177]. In the rhino mouse, utriculi are derived from the infundibular zone of the initial follicular units and are histologically similar to comedones [177]. The rhino mice were treated topically with tretinoin and maxacalcitol once daily for 2 and 4 weeks, respectively. The dermal side of the epidermal sheet was observed to determine the size of the utricle. Hematoxylin and eosin-stained vertical sections were used to measure utricle diameter and density and to evaluate histological changes. Maxacalcitol

(25 µg/g) and tretinoin (0.1%) significantly decreased the size and the diameter of the utricle after 1 week of treatment. Histopathologically, maxacalcitol and tretinoin markedly induced epidermal hyperplasia accompanied by a minor accumulation of inflammatory cells in the dermis, with and without hypercornification, respectively. These results indicate that maxacalcitol has an effect on comedolysis and that its mechanism of action may be different from that of retinoids. The clinical relevance of these observations remains to be elucidated in future investigations.

## 6.10 Cutaneous wound healing and skin health

Because 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs were such potent inhibitors of keratinocyte and fibroblast proliferation, there was concern that its topical application could result in skin atrophy and poor wound healing that is seen with topical steroids. However, Tian et al. [178] observed that the topical application of 1,25(OH)<sub>2</sub>D<sub>3</sub> markedly enhanced wound healing compared with the topical application of a control vehicle or vitamin D<sub>3</sub>. As little as 5–50 ng of topically administered 1,25(OH)<sub>2</sub>D<sub>3</sub> to the wound in rats increased wound healing by 75% on the first day compared with the control group. This accelerated wound healing continued until it was healed.

When the skin is wounded, it triggers an acute inflammatory response with the innate immune system helping to protect against invasive organisms. 1,25(OH)<sub>2</sub>D<sub>3</sub> upregulates the antimicrobial peptide hCAP18/LL-37(cathelicidin) [179] to help reduce infection. The inflow of inflammatory cells into the wounded area results in the release of cytokines and growth factors to initiate proliferation and migration of dermal and epidermal cells. Mice lacking the VDR when placed on a low-calcium diet have a delayed wound healing. This was associated with reduced β-catenin transcriptional activity and proliferation in the cells at the leading edge of wound closure. Thus, it was concluded that vitamin D and calcium signaling are necessary components of the epidermal response to wounding by regulating stem cell activation through increased β-catenin transcriptional activity [180].

Thus 1,25(OH)<sub>2</sub>D<sub>3</sub> is a potent modulator of skin health. Under conditions of hyperproliferation, such as psoriasis and actinic keratoses, this hormone can induce a variety of biologic processes (cytokines and growth inhibitory factors) to help restore normal proliferation and improve skin health. However, if the skin becomes atrophic because of topical steroid use or is wounded, 1,25(OH)<sub>2</sub>D<sub>3</sub> is able to induce other biologic processes (cytokines and growth factors) to restore normal skin growth and enhance wound healing.

## 6.11 Other skin diseases

A number of case reports demonstrate positive effects of topical treatment with vitamin D analogs in a variety of skin diseases such as transient acantholytic dermatosis (Grover's disease), inflammatory linear verrucous epidermal naevus, disseminated superficial actinic porokeratosis, pityriasis rubra pilaris, epidermolytic palmoplantar keratoderma of Vörner, confluent and reticulated papillomatosis (Gougerot–Carteaud syndrome), and Sjögren–Larsson syndrome [181,182].

## 7. Evaluation of new vitamin D analogs, with less calcemic activity, which can be used for the treatment of hyperproliferative skin disorders

The use of vitamin D analogs in dermatology and other medical fields was shown to be limited because serious side effects, mainly on calcium metabolism, may occur at supraphysiological doses needed to reach clinical improvement [183–186]. The evaluation of new vitamin D compounds with strong immunosuppressive, antiproliferative, and differentiating effects, but only marginal effects on calcium metabolism introduce new important therapies for the treatment of various skin diseases. The goal to create new vitamin D analogs with selective biological activity and no undesirable side effects has still not been reached, but recent findings introduce new and promising concepts. Chapters 45 and 46 of this book discuss the development of 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs.

Calcipotriol (MC 903) with similar VDR binding properties compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>, but low affinity for the vitamin D-binding protein (DBP), is well known to be effective and safe in the topical treatment of psoriasis [86]. In vivo studies in rats showed that effects of calcipotriol on calcium metabolism are 100–200 times lower as compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>, whereas in vitro effects on proliferation and differentiation on human keratinocytes are comparable. These differential effects are probably caused by the different pharmacokinetic profiles of calcipotriol and 1,25(OH)<sub>2</sub>D<sub>3</sub> due in part to different affinities for DBP. Serum half-life in rats of these vitamin D analogs was shown to be 4 minutes after treatment with calcipotriol in contrast to 15 minutes after treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> [reviewed in 172]. However, most of the calcium metabolic studies comparing 1,25(OH)<sub>2</sub>D<sub>3</sub> and calcipotriol were done in vivo, whereas most studies analyzing proliferation or differentiation were done in vitro. The rapid degradation of calcipotriol after systemic administration has limited its oral use but made it an ideal drug for topical use.

A different approach to create new vitamin D analogs that are effective in the topical treatment of

hyperproliferative or inflammatory skin diseases is the goal to create new synthetic compounds that are metabolized in the skin and therefore exert only limited systemic side effects. Vitamin D analogs, obtained by a combination of the 20-methyl modification with biologically interesting artificial side chain subunits [184] or 2 $\beta$ -substituted 1,25(OH) $_2$ D $_3$  [185], are promising candidates for this approach.

Another interesting approach to enhance the local concentration of 1,25(OH) $_2$ D $_3$  in the skin without obtaining systemic side effects is an attempt to specifically inhibit the activity of vitamin D–metabolizing enzymes, i.e., various hydroxylases (catabolic D $_3$ -OHases for 1,25(OH) $_2$ D $_3$ ) that are present in the skin and that are responsible for the catabolism of 1,25(OH) $_2$ D $_3$  [186]. It is known that various pharmacologic active compounds, including not only other steroidal hormones but also cytochrome P450 inhibitors as ketoconazole, inhibit the activity of vitamin D hydroxylases in the skin [187]. It may be possible to locally enhance the concentration of endogenous calcitriol in the skin by the topical application of these compounds without obtaining systemic side effects. It can be speculated that the therapeutic effects of various antimycotic compounds including ketoconazole in the treatment of seborrheic dermatitis may at least in part be because of this mechanism. It is now known that VDR requires nuclear accessory proteins for efficient binding to vitamin D response elements in promoter regions of target genes, thereby inducing VDR-mediated transactivation [186]. As a consequence, different vitamin D analogs may have (depending on their chemical structure) different affinities for the various homo- or heterodimers of VDR and nuclear cofactors including RXR $\alpha$  [188,189]. The synthesis of new vitamin D analogs that activate different vitamin D signaling pathways may lead to the introduction of new therapeutics for the topical or oral treatment of various skin diseases. These new drugs may induce strong effects on target cell proliferation and differentiation in the skin or the immune system but only marginal effects on calcium metabolism. Another approach to enhance the therapeutic effects of orally or topically administered 1,25(OH) $_2$ D $_3$  may be the combination with synergistic acting drugs. The discovery of different vitamin D signaling pathways that are determined and regulated by cofactors of VDR including RXR $\alpha$  and their corresponding ligands suggests that 9-cis RA or all-trans RA may act synergistically with vitamin D analogs to induce VDR-mediated transactivation and regulate the transcriptional activity of distinct gene networks [188,189]. Only a little is known about the effects of the combined application of vitamin D and vitamin A analogs under physiological or pathophysiological conditions *in vivo* [188,189]. This combination may

selectively enhance or block different biological effects of vitamin D analogs that are mediated by different vitamin D signaling pathways.

## 8. Conclusion

It can be speculated that innovative vitamin D analogs will introduce novel alternatives for the treatment of various skin disorders. If the final goal to create strong antiproliferative and antiinflammatory vitamin D analogs with only minor calcemic activity is reached, these new agents would herald a new era in dermatologic therapy, which possibly can be compared with the introduction of synthetic corticosteroids or retinoids. These new drugs may activate selective vitamin D signaling pathways, but may exert only negligible calcemic activity, and may also be effective in the systemic treatment of various skin malignancies, including lymphomas, cutaneous squamous cell carcinomas (SCCs), or basal cell carcinomas (BCCs). Unfortunately, although thousands of analogs of 1,25(OH) $_2$ D $_3$  have been produced, most have not satisfied all the desired criteria, i.e., having prolonged potent therapeutic activity while at the same time having no toxic consequences on calcium and bone metabolism. One promising avenue of investigation is the resurrection of the use of very high doses of vitamin D. Although the pioneering studies in the 1930s–1950s using high-dose vitamin D to treat psoriasis demonstrated efficacy, it also resulted in significant toxicity mainly because of lack of understanding, at that time, how vitamin D functioned on calcium metabolism and that it required further metabolism. To mitigate the toxic calcium–related side effects of high-dose vitamin D, Finamor [94] developed a protocol using high-dose vitamin D $_3$  with strict guidelines to eliminate all calcium from the diet to treat a variety of autoimmune disorders, including psoriasis and vitiligo. Recently, it was observed that very high doses of vitamin D daily for 10 days were effective in treating congenital ichthyosis. Therefore, there continues to be promised for the use of 1,25(OH) $_2$ D $_3$  and its analogs as well as vitamin D $_3$  itself in high doses for the treatment of a variety of an inherited and acquired skin disorders.

## 9. Summary points

- Provides the reader with a historical perspective on the use of vitamin D $_3$  and active vitamin D $_3$  and its analogs in dermatology.
- Provides the reader with an understanding of how 1,25-dihydroxyvitamin D $_3$  and its active analogs

affect the immune system and keratinocyte proliferation and differentiation.

- Provides the reader with information on the clinical utility of 1,25-dihydroxyvitamin D<sub>3</sub> and its analogs for the treatment of psoriasis.
- Provides the reader with up-to-date information on the clinical use of 1,25-dihydroxyvitamin D<sub>3</sub> for the treatment of ichthyosis.
- Provides the reader with an understanding of how to safely use pharmacologic doses of vitamin D<sub>3</sub> to treat psoriasis, vitiligo, and ichthyosis.

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## Further reading

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# Vitamin D, acute respiratory infection, and Asthma/COPD

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## OBJECTIVES

- The chapter will describe how vitamin D plays an important role in regulating innate and adaptive immune responses that are central to normal respiratory function.
- The chapter will outline how vitamin D deficiency has been linked to common respiratory disorders including asthma and chronic obstructive pulmonary disease (COPD).
- Observational and interventional studies of vitamin D and acute respiratory infection (ARI), asthma, and COPD will be detailed.

## 1. Introduction

For most of the 20th century, medical research on sunlight and vitamin D focused on calcium-phosphate homeostasis and bone health, particularly the prevention and treatment of rickets. Nevertheless, one of the first Nobel Prizes in Physiology and Medicine was awarded in 1903 to Dr. Niels Ryberg Finsen for his discovery of the beneficial effects of sunlight on cutaneous tuberculosis (TB) [1]. This novel *infectious disease* finding led to important changes in the clinical management of pulmonary TB worldwide, including the development of mountain clinics to provide afflicted patients with better access to sunlight and fresh air [2]. Although the link between sunlight exposure and vitamin D synthesis, and between vitamin D deficiency and TB, was widely

appreciated during the first half of the 20th century, the role of sunlight and vitamin D on respiratory infection was largely neglected during the latter half of the 20th century.

Over the past 25 years, however, researchers around the world have again taken interest in the antimicrobial potential of sunlight and vitamin D [3,4]. For example, while initial studies in the 1990s demonstrated an association between nutritional rickets and ARI among children in developing nations [5,6], many studies since have found an association between vitamin D and ARI risk in otherwise well-nourished individuals, of all ages, in many developed nations [7,8]. Research has progressed rapidly, from small cross-sectional and case-control studies to large prospective cohort studies and randomized controlled trials (RCTs).

With growing evidence that the vitamin D hormone has complex immunologic effects, it was not surprising that some investigators raised health concerns about this supplementation. Most notably, Wjst and Dold proposed in 1999 that vitamin D supplementation might be a *cause* of the global increases in asthma and other allergic disorders [9]. Genetic association studies were undertaken to address this putative harm by examining if specific polymorphisms of the vitamin D receptor (VDR) gene were associated with an increased risk of asthma. Although these asthma genetics studies showed some statistically significant associations, the results conflicted across individual datasets and studies [10–13]; the relationship between VDR polymorphisms and asthma remained unclear.

In this context, Camargo et al. proposed the *opposite* hypothesis of Wjst et al. at the 2006 Annual Meeting of the American Academy of Allergy, Asthma, and

Immunology [14]—i.e., that widespread vitamin D *deficiency* (not excess) might explain recent increases in childhood wheezing and asthma. The investigators presented original evidence for this hypothesis from a Boston birth cohort study involving almost 1200 mother-child pairs. In brief, the investigators found that maternal intake of vitamin D during pregnancy had a striking inverse association with the risk of recurrent wheezing in offspring. Because most wheezing illness of childhood represents uncomplicated ARI, rather than incident asthma [15], the authors cautioned that further research would need to distinguish between simple ARIs (with consequent wheezing) versus a true diagnosis of childhood asthma.

Over the past 15 years, the relation of low vitamin D status with ARIs and asthma has become a focus of research teams worldwide. Indeed, major professional society meetings today, in both the pulmonary and allergy/immunology communities, often include lectures or entire symposia on vitamin D. This chapter examines the relation of vitamin D to childhood wheezing and asthma, along with emerging evidence on the role of vitamin D among adults with COPD. To decrease overlap with other parts of [Section 11](#) of this book on immune function and inflammation (notably Chapter 96), this chapter will touch only briefly on the immunologic mechanisms that likely mediate these clinical and epidemiological observations. Likewise, this chapter will touch only briefly on the emerging evidence on the relation of vitamin D with atopy and allergic diseases (such as atopic dermatitis, food allergy, and allergic rhinitis), given their mechanistic links with childhood asthma [16].

## 2. Common respiratory disorders

To better understand the effect of vitamin D on common respiratory disorders, it is important to discuss, at least briefly, some of the methodological challenges of research in this area, especially the complex interrelations between these disorders. This section provides key background information on respiratory infections, childhood wheezing, asthma, and COPD. The text then returns to the role of vitamin D in each of these disorders.

### 2.1 Respiratory infections

Respiratory infections are a diverse and complex group of infections that have always been a major cause of morbidity and mortality [17,18], as demonstrated by the COVID-19 pandemic. Although these infections are frequently categorized anatomically into *upper* versus *lower* respiratory tract infections, respiratory infections

may (and often do) involve multiple anatomic locations. Another method of categorization is according to a pathogen, with infections commonly divided into *viral* versus *bacterial*. For some infections with respiratory involvement, researchers and the general public commonly identify them by the *specific pathogen* alone, as is true for influenza and TB. There also are basic issues of disease chronicity (i.e., *acute* vs. *chronic* infection). As a result of these and other considerations, clinical and epidemiologic research on the relationship between vitamin D and “respiratory infections” has proven quite challenging.

Bronchiolitis provides an excellent example of the complexity of research on ARIs. Although bronchiolitis is the leading cause of hospitalization in infants and has high annual costs [19], there is no global consensus regarding its definition. Because bronchiolitis remains a somewhat vague clinical diagnosis, it should not surprise readers that clinicians differ on who exactly has the illness [20]. In 2006, an American Academy of Pediatrics position paper described the typical child with bronchiolitis as being age <2 years and having “rhinitis, tachypnea, wheezing, cough, crackles, use of accessory muscles, and/or nasal flaring” [21]; the definition did not materially change in the updated 2104 guidelines [22]. In other countries, however, many investigators believe that bronchiolitis *only* should be diagnosed in infants (defined as age <1 year), or that the presence of specific exam findings (e.g., crackles) should be mandatory.

Although the microbiology of bronchiolitis might help with classification, there is a spectrum of clinical disease that is not understood. For example, while almost all children are infected by age 2 years with respiratory syncytial virus (RSV) [23,24], the leading cause of severe bronchiolitis [25–27], most children infected with RSV do not present to an acute care setting with clinical bronchiolitis [23,28]. Indeed, <10% of US children infected with RSV will present to the emergency department with bronchiolitis, and only 2%–3% of all children are hospitalized [27,29,30]. To further complicate matters, not only is there a growing list of other viruses linked to bronchiolitis ([Table 105.1](#)), including rhinovirus [27,31,32], but it is clear that multiple pathogen infections (co-infections) are common [33,34]. Even without considering the different short-term and long-term clinical implications of these different forms of bronchiolitis [35], one can quickly understand the complexity of classifying, let alone studying, this common ARI of childhood.

In summary, the heterogeneity of “respiratory infection” makes it challenging to link an individual factor like vitamin D to the risk of developing this composite outcome. To be more specific, vitamin D might have an important association with some, but not all, types

**TABLE 105.1** Frequency of common viruses linked to bronchiolitis, according to healthcare setting.

	Outpatient <sup>a</sup>	Emergency <sup>b</sup>	Inpatient <sup>c</sup>
Respiratory syncytial virus	11%–27%	64%	73%
Rhinovirus	46%–49%	16%	26%
Parainfluenza	5%–13%	n/a	3%
Influenza	1%–5%	4%	1%
Metapneumovirus	2%	7%	7%
Combination infections	10%–17%	14%	30%

<sup>a</sup>Based on upper and lower acute respiratory infections during infants' first winter [195] and first year [196].

<sup>b</sup>Based on children presenting to 14 U.S. emergency departments [197].

<sup>c</sup>Based on children admitted to 16 U.S. hospitals [33].

Abbreviation: n/a denotes not available.

of respiratory infections, as defined by either specific anatomical areas or by specific pathogenic organisms. Vitamin D also might influence disease severity. For example, while all children may at some point be infected with RSV, perhaps only those with low serum 25-hydroxyvitamin D (25(OH)D) levels—or other predisposing factors—develop severe enough RSV infections to require hospitalization or intensive care. If these different hypotheses are true, the common practice of collapsing diverse infections into one composite “respiratory infection” outcome would likely obscure real associations—i.e., one would conclude that there was no association when, in fact, there was. Studies on the relation of sunlight and vitamin D status to ARI (and associated disorders) need to be interpreted with this important caveat in mind.

## 2.2 Childhood wheezing

The heterogeneity of respiratory infections is similar to that of childhood wheezing. After all, many different conditions can cause “wheezing,” which is just a musical sound caused by the passage of air through narrowed respiratory tract airways [36]. Across the lifespan, this airway narrowing probably is caused most often by ARIs. For individuals with asthma or COPD, however, the airway narrowing and associated wheezing could result from a complex mixture of either baseline or exacerbated inflammation/edema and bronchoconstriction, often but not always with concurrent infection. In all ages, wheezing also can be caused by other, less common conditions. A classic cause of childhood wheezing unrelated to asthma is an airway foreign body [37]. Among adults, decompensated heart failure—with so-called cardiac wheezing—is common asthma/COPD mimic in the acute care setting [38].

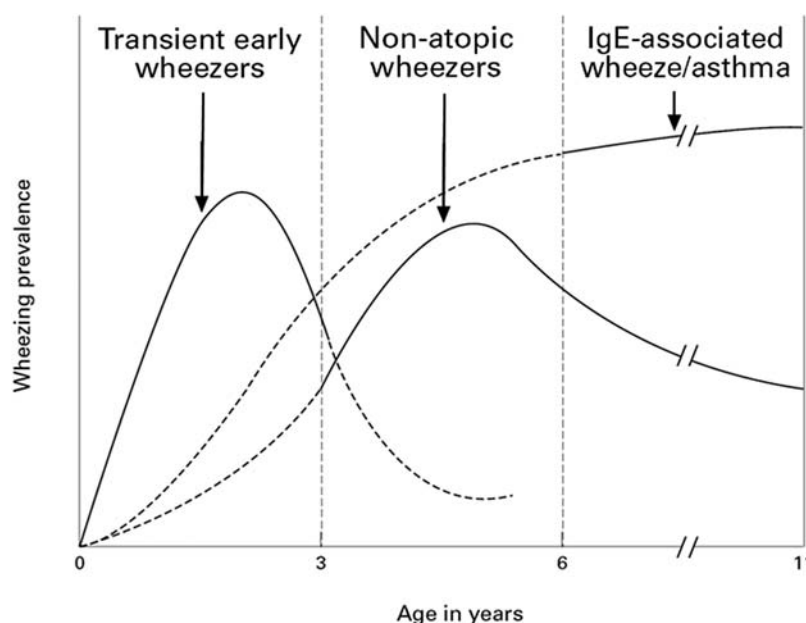
Population-based cohort studies have provided important information about the natural history of childhood wheezing. For example, the landmark studies of Martinez et al. clearly demonstrate that many children who wheeze in early childhood have only transient episodes (during ARIs) and do *not* go on to develop asthma [15,39]. Accordingly, asthma researchers try, with varying levels of success, to divide wheezing children into different clinical groups. In one system, children are grouped into (1) transient early wheezing, (2) late-onset wheezing, or (3) persistent wheezing (i.e., when the wheezing was present during both early and late periods of early childhood). In another system, children are grouped into: (1) transient early wheezers, (2) nonatopic wheezers, or (3) IgE-associated wheeze/asthma (Fig. 105.1). Although these groupings help to divide children according to their likelihood of developing asthma by age 6 years, many children—in all groups—do *not* develop asthma. Studies have repeatedly affirmed the clinical adage that “All that wheezes is not asthma.” To further emphasize the difference between childhood wheezing and asthma, many young children with asthma present with recurrent nocturnal cough and lack any evidence of wheezing [40]. For all of these reasons, one should be very cautious about generalizing from findings about childhood wheezing to actual asthma. This cautionary note applies even to *recurrent* wheezing, which may simply represent recurrent ARIs in a nonasthmatic child. Since young children are estimated to experience 6 to 10 ARIs per year [41], clinicians (and researchers) might anticipate that many attentive parents may report recurrent “wheezing” in their children and that this could be mistakenly called asthma.

## 2.3 Asthma

Asthma is a common medical condition that is associated with high morbidity and health care utilization [42]. Diagnosis and management guidelines are available from both the U.S. National Institutes of Health [43] and an international group called the “Global Initiative for Asthma” [44]. Although defining asthma challenged clinicians for centuries, there now is general acceptance of the 1987 definition from the American Thoracic Society (ATS) [45]—i.e., asthma is a chronic lung disease characterized by: (1) airway narrowing that is reversible (though not always completely), either spontaneously or with treatment; (2) airway inflammation; and (3) bronchial hyper-responsiveness to a variety of stimuli.

Although the ATS asthma definition is clear, it has proven very difficult to apply in epidemiologic studies, where subjects may be dispersed over large





**FIGURE 105.1** Hypothetical prevalence of three different wheezing phenotypes in childhood. For each age interval, the overall wheezing prevalence is the sum of the areas under each curve. The dashed lines emphasize the possibility of different curve shapes due to many factors, including overlap between the three groups. Adapted from Ref. [194].

geographic areas and where spirometry and hyper-responsiveness testing usually are not feasible. As a result, asthma epidemiologists have relied on much simpler definitions of asthma, such as an affirmative response to the question: “Do you have doctor-diagnosed asthma?” Although the potential limitations of this epidemiologic approach are self-evident, this approach actually performs with adequate accuracy [46] to allow epidemiologists to perform asthma surveillance and to examine potential risk factors for the disease. Similar questions have, in fact, contributed to widespread concern about the dramatic rise in “asthma” prevalence for several decades [47].

While viral ARIs are common triggers of asthma exacerbations [48,49], and these exacerbations occur across the lifespan, the vast majority of asthma begins in early childhood. Estimates vary but approximately 80%–90% of asthma begins before age 6 years, with 70% of asthmatic children having asthma symptoms before age 3 years [50,51]. The etiology of asthma has proven elusive, but these clinical observations indicate that the major risk factors must be present in early life—either in utero or at least in the first months/year of childhood [52]. Childhood atopy is one of the strongest risk factors for asthma [16,53], yet substantial numbers of asthmatic individuals are non-atopic [54]. Indeed, a growing number of asthma researchers acknowledge that the term “asthma” is likely a *syndrome* composed of heterogeneous diseases [55], with significant overlap with COPD [56]; the etiologic factors and treatments of one type of asthma may differ from those of another.

## 2.4 Chronic obstructive pulmonary disease

COPD is another common medical condition that is associated with high morbidity and mortality [57]. The most widely accepted definition for COPD comes from the “Global Initiative for Chronic Obstructive Lung Disease” (GOLD): COPD is “a common, preventable and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases and influenced by host factors including abnormal lung development. Significant comorbidities may have an impact on morbidity and mortality.” [57].

Even more so than for asthma, there have been significant disagreements about the definition of COPD over the past 50 years. Classical descriptions of COPD emphasized two major types: “chronic bronchitis” and “emphysema.” Although these COPD types continue to be taught to healthcare professionals, there now is universal recognition of their considerable overlap. Uncertainty regarding COPD diagnosis has been compounded by the fact that chronic bronchitis was, for many years, a *clinical* diagnosis (e.g., requiring that the patient have a daily cough productive of sputum for at least 3 months over at least two consecutive years) while emphysema was an *anatomic* diagnosis requiring actual parenchymal destruction. By contrast, the GOLD guidelines acknowledge that the chronic airflow limitation characteristic of COPD is caused by a mixture of small airway disease (obstructive bronchiolitis) and

parenchymal destruction (emphysema), with the relative contributions of these pathological changes varying from person to person [57]. The evolving definition of COPD requires careful attention to case definition when integrating study results across recent decades. As with asthma, the etiologic factors and treatments of one type of COPD may differ from those of another.

Unlike asthma, which usually starts in early childhood but can begin in adulthood (e.g., occupational asthma), COPD manifests much later in life, typically after decades of tobacco smoke inhalation [57]. Moreover, while both asthma and COPD are “obstructive lung diseases” characterized by virus-induced exacerbations, acute exacerbations of COPD (AECOPD) are more heterogeneous than those of asthma; this has several explanations but one reason is that elderly smokers are likely to suffer from other health problems (e.g., cardiovascular disease) and these can contribute to the presentation and clinical course of AECOPD. Thus, while it is tempting for researchers to generalize vitamin D-related findings from asthma to COPD, or vice versa, this should be done cautiously given important differences between these separate, but overlapping, obstructive airway disorders.

### 3. Vitamin D and lung development

Although the physiologic role of vitamin D in the developing fetus, neonate, and infant is discussed in Chapter 33, lung development is so critical to the development of asthma and COPD that it merits special comment here. Briefly, human lung development begins around 4 weeks gestation and the bronchi and major branches develop in the first few months of fetal growth. The development of alveolar units probably continues after birth, but it is generally believed that branching of the airways does not. Although there are limited data about the role of vitamin D in human lung development, rat models may provide insights into some of the critical steps in lung development across species. For example, pulmonary surfactant is a mostly phospholipid complex present in the alveolus to reduce surface tension and regulate innate immune function, and it is critical to lung development [58]. In a 1990 publication, Marin et al. demonstrated, in explanted fetal rat lung tissue, that 1,25-dihydroxyvitamin D ( $1,25(\text{OH})_2\text{D}$ ) stimulates the synthesis of pulmonary surfactant phospholipids and the release of surfactant from alveolar type II (AT II) cells [59]. A 1996 publication by the same group showed that fetal rat lung fibroblasts in late pregnancy produce  $1,25(\text{OH})_2\text{D}$ , which binds to numerous VDRs on AT II cells and thereby stimulates the production of pulmonary surfactant [60]. These findings led the authors to propose the existence of a vitamin

D paracrine system that regulates the maturation of the rat lung.

While these rat lung data are intriguing, their relevance to humans is uncertain. For example, a 2005 study of human fetal lung tissue demonstrated that  $1,25(\text{OH})_2\text{D}$  does *not* induce the expression of surfactant proteins in a coordinated manner [61]. Moreover, lung development requires many other complex steps, including thinning the alveolar septal wall to allow effective gas exchange [62]. This thinning is thought to occur via apoptosis of lung fibroblasts [63]. Sukurai et al. explored this issue in fetal rat lungs and found that  $1,25(\text{OH})_2\text{D}$ , acting via local effects, promotes alveolar epithelial-mesenchymal interactions and inhibits lipofibroblast atoptosis [64]. Moreover, the investigators found that rat pups administered  $1,25(\text{OH})_2\text{D}$  postnatally showed increased expressions of key lipofibroblast and AT II cell differentiation markers, decreased spontaneous alveolar lipofibroblast and ATII cell apoptosis, increased alveolar count, and increased septal thickness. Work by Nadeau et al. also suggests complex effects of  $1,25(\text{OH})_2\text{D}$  on rat lung development during the postnatal period [65]. These investigators demonstrated a complex integration of the effects of glucocorticoids, retinoic acid, and  $1,25(\text{OH})_2\text{D}$  on gene expression in the postnatal lung, specifically *Lgl 1*; the authors concluded that all three hormones probably contribute to the timely advance of alveolarization without attendant inflammation.

In 2011, Zosky et al. reported on their mouse model of vitamin D deficiency, in which they had studied offspring from both vitamin D deficient and vitamin D replete colonies of mice at 2 weeks of age [66]. The investigators found that vitamin D deficiency caused lung function deficits that were primarily explained by differences in lung volume. In 2014, Yurt et al. used an in vivo rat model to determine the pulmonary effects of perinatal vitamin D deficiency and reported compelling evidence for the potential role of vitamin D supplementation in asthma prevention [67]. Briefly, the investigators found that perinatal vitamin D deficiency altered airway contractility and that this effect was not seen in the offspring of maternal rats given vitamin D supplementation. Most recently, in 2021, Sakurai et al. reported in their established rat model that perinatal vitamin D deficiency alters lung mesenchymal cell proliferation and differentiation [68]. These observations lend support to vitamin D deficiency as a contributing factor to the increased respiratory morbidity seen in children born to mothers with vitamin D deficiency.

In sum, studies over the past 30 years support the presence of a vitamin D paracrine system in both the fetal and postnatal rat lung and the potential for asthma/COPD prevention. While much remains to be learned about the connection of vitamin D to human

lung development, it seems likely that vitamin D is playing some role in this complex process. Very low or very high levels of vitamin D—either locally or systemically—might have lasting adverse effects on lung development and function. Although speculative, such effects would provide a mechanism for how gestational and even postnatal vitamin D status could influence respiratory health.

## 4. Vitamin D and acute respiratory infection

As noted earlier, the first clear link between vitamin D and infectious disease goes back more than a century to the TB research of Dr. Finsen, who discovered that concentrated light radiation was an effective treatment for lupus vulgaris (cutaneous TB) [1]. Although he was not aware of the mechanism for this therapeutic benefit, scientists today would attribute his treatment successes, at least in part, to UVB-induced increases in vitamin D. For the past 20 years, investigators around the world have focused on the link between vitamin D status and chronic infection with TB. Observational studies have provided promising evidence for an inverse association between vitamin D and TB [69], but more recent RCTs have not shown benefits [70,71]. Global investigators have also investigated, more generally, the link between sunlight, vitamin D, and a variety of infectious diseases [4]. The effect of vitamin D on ARI may be distinct from TB or infections in general. Either way, it is central to understanding the relation of vitamin D with asthma and COPD. The underlying immunologic mechanisms are discussed in more detail in Chapters 94–99.

### 4.1 Observational studies on ARI, including childhood wheezing

Over the past 25 years, many researchers have repeatedly observed that vitamin D appears to have antimicrobial activity, not only against TB but against a broad range of more acute respiratory pathogens. Several early studies showed that nutritional rickets was associated with a higher risk of ARIs [5,6,72] and worse treatment outcomes [73]. However, the relationship between low but nonrachitic 25(OH)D levels and ARI was less clear. While many countries now have a low population prevalence of rickets, they continue to have high rates of vitamin D deficiency [74,75] so the question remains one of major public health importance.

In a 2013 systematic review, Jolliffe et al. identified 25 eligible observational studies (4 cross-sectional, 8 case-control, 13 cohort) on vitamin D and ARI [8]. The authors found that these studies predominantly reported statistically significant associations between low vitamin

D status and increased risk of both upper and lower ARI. The studies involved large numbers of participants of all ages in diverse geographical settings. Moreover, they had a wide distribution of serum 25(OH)D concentrations. Cohort studies were plentiful and thereby confirmed that vitamin D deficiency truly preceded the onset of ARI; reverse causality was highly unlikely. Over the past decade, many more observational studies have supported these conclusions. In a recent meta-analysis by Pham et al. [76], the investigators reported an inverse, nonlinear association between 25(OH)D level and the risk of ARI. They observed that the sharpest increase in ARI risk occurred at 25(OH)D levels less than 15 mg/mL (37.5 nmol/L).

Because most *childhood wheezing* is caused by ARI and is not indicative of actual asthma [15,39], it seems most appropriate to review the observational studies on vitamin D and childhood wheezing in this ARI section. (By contrast, because most RCTs of maternal/infant vitamin D supplementation are clearly aimed at the primary prevention of childhood asthma, they are reviewed in the “Vitamin D and Asthma” section that follows). In 2005, Camargo et al. started to investigate the relation between vitamin D status and childhood wheezing in a Massachusetts birth cohort called Project Viva [77]. The investigators found that lower maternal intake of vitamin D during pregnancy was associated with significantly increased offspring risk of recurrent wheezing. In these 1194 mother-child pairs, the mean (SD) total vitamin D intake during pregnancy was 548 [78] IU/day. By age 3 years, 186 children (16%) had recurrent wheeze. Compared with mothers in the lowest quartile of daily intake (median: 356 IU), those in the highest quartile (724 IU) had substantially lower odds of having a child with recurrent wheeze (OR 0.39; 95% CI 0.25–0.62; P for trend <0.001). An increase in vitamin D intake was associated with lower odds, regardless of whether vitamin D was from diet or from supplements. Adjustment for 12 potential confounders, including maternal intake of other dietary factors, did not change the results. Although the “respiratory infection” outcome was crude, it also showed an inverse association and favored an infectious disease explanation for the study results [77].

The Boston findings on maternal intake of vitamin D and childhood wheezing were replicated in a Scottish birth cohort by Devereux et al. [79]. In these 1212 mother-child pairs, the median (interquartile range) vitamin D intake during pregnancy was 128 [80–142] IU/d, which was significantly lower than the intake of the Boston mothers [77]. Nevertheless, compared to mothers in the lowest quintile of daily intake (median: 77 IU), those in the highest quintile (275 IU) had a lower risk for ever wheeze (OR 0.48; 95%CI 0.25–0.91), wheeze in the previous year (OR 0.35; 95%CI 0.15–0.83), and

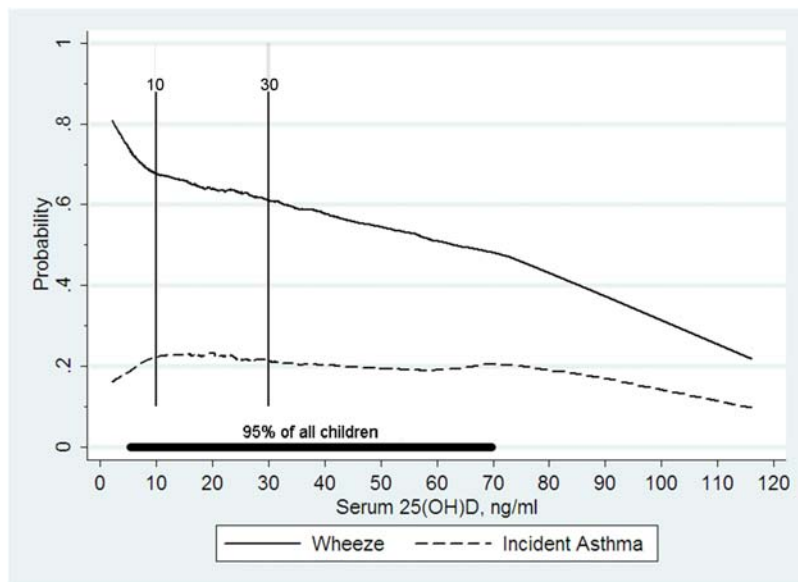
persistent wheeze (OR 0.33; 95%CI 0.11–0.98) in the 5-year-old children. In contrast to these impressive vitamin D-wheeze findings, there was no association between maternal vitamin D intake during pregnancy with doctor-diagnosed asthma at age 5 years ( $P = .98$ ).

Since the 2007 publication of these two studies [77,79], many cohorts from around the world have examined these same questions, using diverse measures of vitamin D status and definitions of wheezing (and early childhood “asthma”). A 2011 systematic review by Nurmatov et al. identified a total of eight eligible observational studies on maternal vitamin D status during pregnancy and offspring risk of childhood wheezing/asthma [143], including the Boston and Scotland cohorts presented above. Pooled analysis of the four large cohort studies showed that higher maternal intake of vitamin D during pregnancy was associated with reduced odds of wheezing, either recurrent or wheeze in the previous year (OR 0.56; 95%CI, 0.42–0.73). Pooled analysis from the two available cohort studies [79,144] showed that maternal vitamin D intake was not associated with asthma at age 5 years ( $P = .42$ ). These important findings (inverse association with wheezing but no association with actual asthma) were affirmed in a 2015 systematic review by Beckhaus et al. [145].

In the New Zealand Asthma and Allergy Cohort Study, Camargo et al. first examined the association between low cord blood levels of 25(OH)D and subsequent risk of ARIs and childhood wheezing [146]. The availability of 25(OH)D levels provided a distinct advantage to prior studies since it better-assessed vitamin D status than self-reports of oral intake alone. In this birth cohort

of 922 children with cord blood specimens available for assay, 20% of children had cord blood 25(OH)D level  $<10$  ng/mL, and 73% (cumulative) had a 25(OH)D level  $<30$  ng/mL. Cord blood 25(OH)D level was inversely associated with odds of ARI by age 3 months: children with 25(OH)D levels  $<10$  ng/mL were at a twofold higher odds than those with levels  $\geq 30$  ng/mL (OR 2.04; 95%CI 1.13–3.67). These prospective findings were independent of season and 14 other potential confounders and provided strong evidence for a protective effect of vitamin D on the risk of ARI. Indeed, cord blood 25(OH)D level also had an inverse association with risk of wheezing illness at ages 15 months, 3 years, and 5 years (all  $P < .05$ ; see Fig. 105.2). By contrast, cord blood 25(OH)D level had no association with doctor-diagnosed asthma at age 5 years ( $P = .37$ ).

Over the past decade, several groups from around the world have confirmed the inverse association between maternal and early childhood vitamin D status and risk of ARI/wheeze. For example, Saraf et al. performed a nested case-control study in large prebirth cohort in New Zealand [147] and found that birth 25(OH)D levels  $<20$  ng/mL were associated with significantly elevated odds of ARI hospitalization during infancy (OR 2.20; 95%CI, 1.48–2.91). Nevertheless, some studies have not found the inverse association. For example, a 2015 study by Visness et al. found no association between cord blood 25(OH)D level and risk of childhood wheezing in two well-established U.S. birth cohorts [148]. While this important null study does not negate the prior studies showing an inverse association, it does challenge all investigators to identify possible



**FIGURE 105.2** Associations between cord blood 25-hydroxyvitamin D (25[OH]D) levels with probabilities of cumulative wheeze or incident asthma by age 5 years ( $n = 922$  children in New Zealand). The vertical lines denote serum 25(OH)D benchmarks (in ng/mL). Adapted from Ref. [146].



effect modifiers that might explain the discrepant results. In other words, what environmental and/or genetic differences were there between the different study populations that might explain these strikingly different results?

Along those lines, relatively few groups have examined the effect of vitamin D pathway-related polymorphisms on the association between vitamin D status and ARI. A 2014 systematic review by McNally et al. identified three eligible studies on VDR polymorphisms and severe RSV bronchiolitis [149]. Unfortunately, only two VDR polymorphisms were included in more than one study: FokI (rs2228570) and TaqI (rs731236). All three studies reported a positive association between the FokI minor allele and disease (OR 1.52, 95%CI 1.12–2.05). Although statistical power was limited, the genotype analysis suggested a dominant or incomplete dominance model with combined ORs for fF (OR 1.73, 95%CI 0.92–3.36) and ff (OR 2.24, 95%CI 0.98–5.14) compared to the FF genotype. By contrast, the authors did not observe an association between TaqI and severe RSV-bronchiolitis at the allele or genotype level. In another study, Randolph et al. reported on the association between vitamin D-binding protein (DBP) haplotypes and severe RSV bronchiolitis [150]. Briefly, the study involved 198 children hospitalized with RSV bronchiolitis in Boston (and their 333 parents) and then was validated in a larger sample from the Netherlands. The investigators found that GC1s haplotype carriage may increase the risk of RSV bronchiolitis and subsequent asthma development. They noted that this haplotype is associated with higher DBP levels, perhaps resulting in less freely available 25(OH)D. More recently, Zacharioudaki et al. reported a prospective case-control study of infants with bacterial ( $n = 40$ ) or viral ( $n = 52$ ) infection, and 40 age-matched healthy controls [151]; most of the infections were respiratory. Compared to controls, infants with viral infection were more likely to have TaqI polymorphism, t allele (OR 1.96; 95%CI, 1.1–3.58), while controls were more likely to have the haplotype Gc1F of VDBP (OR 2.7; 95%CI, 1.3–5.6); no significant differences were found for the bacterial infection group, nor CYP27B1 (rs10877012) for either the viral or bacterial groups.

Although all of these genetic findings require confirmation, they suggest that an improved understanding of the interplay between vitamin D status and vitamin D pathway-related polymorphisms may influence our interpretation of studies on vitamin D and ARI/wheezing. Likewise, failure to control, or otherwise account, for these genetic factors may tend to obscure real differences between groups. VDR polymorphism data are discussed further in Chapters 60 and 61 and DBP polymorphisms in [Chapters 7 and 60](#).

Finally, on a different genetic tangent, Colak et al. recently used Mendelian randomization (MR) in two population-based cohorts to examine the relation of low vitamin D status with a specific type of ARI, bacterial pneumonia [152]. With >110,000 individuals and up to 38 years follow-up, they discovered that low plasma 25(OH)D (<10 ng/mL) was associated—both observationally and genetically—with an increased risk of bacterial pneumonia. While not RCT-level evidence, this MR analysis suggests a causal inverse association. The application of MR analysis in studying the effects of vitamin D status across the lifespan is discussed in greater detail in Chapter 61.

## 4.2 Interventional studies on ARI

The first interventional studies on vitamin D supplementation and ARI emerged during the 1990s and early 2000s. For example, one study gave 600–700 IU of vitamin D daily from cod liver oil/multivitamin supplementation [153] while another gave 60,000 IU weekly from a vitamin D/calcium supplement [154]; both studies noted less ARIs among children receiving supplementation. Secondary analyses of two RCTs on vitamin D supplementation for bone health also suggested a possible antiinfective benefit [155,156]. Since these early efforts, dozens of RCTs have directly focused on the effect of vitamin D supplementation (vs. placebo) on the risk of ARI. Accordingly, it should now be possible to go beyond the epidemiological associations in the reviewed observational studies and start to draw causal inferences.

Unfortunately, the design and interpretation of RCTs on any nutrient [157], including vitamin D supplements [158], presents major challenges. In addition to traditional concerns about trial participants (e.g., age, baseline vitamin D status, race/ethnicity, body mass index, comorbidities, genetic factors) and sample size, vitamin D trials need to commit to an “optimal” *vitamin D regimen* (frequency, initial bolus dose or not, regular dose, and duration). Although RCTs typically assign a uniform dose to subjects (as compared to placebo), vitamin D trials probably would be more effective with an individualized approach that strived for an optimal level of serum 25(OH)D. Logistical challenges, however, preclude this approach because real-time measurement of baseline 25(OH)D levels is likely to reveal vitamin D deficiency, which would, for ethical reasons, require treatment with vitamin D, particularly in a trial of long duration; such a cointervention would seriously impair the ability of the RCT to demonstrate any vitamin D effects. Exclusion of all subjects with vitamin D deficiency would, of course, make it impossible to study the effect of vitamin D supplementation in that target

population! With regard to frequency, while daily dosing seems more physiologic, monthly dosing is likely to have better adherence, an important consideration in trials of many years duration. Likewise, RCTs probably need to be of sufficient duration for serum levels of 25(OH)D to stabilize and, consequently, for vitamin D to exert its full effect. Shorter trials (e.g., those <6 months) may indeed show clinical differences but they would do so despite a 2- to 3-month delay in the treatment group reaching the 25(OH)D level that might be necessary for the hypothesized benefits.

Another important consideration is the comparison group [158]. Is it a true placebo (i.e., no vitamin D supplement or change in sunlight exposure) or is it placebo with “allowance” of vitamin D usage (e.g., up to 800 IU/day)? The latter may be required for ethical reasons in trials of longer duration but would create a challenge if the lower dose is sufficient to obtain the hypothesized benefit? Does the trial report a vitamin D supplement versus placebo comparison but, in reality, the placebo group improves their vitamin D status outside of the trial protocol. This placebo group “contamination” is particularly challenging since participants can simply go out in the sun for 10–15 min a few times weekly and thereby create large amounts of vitamin D. Clearly, this presents a unique challenge when testing the therapeutic effects of a vitamin D supplement!

With these caveats in mind, the emergence of multiple RCTs on the effect of vitamin D supplementation on ARI risk led to multiple systematic reviews on the topic [8,159,160]. These reviews have consistently agreed that vitamin D supplements appear safe and well-tolerated. With regard to their effect on ARI risk, most review support benefits but also comment on the extreme heterogeneity across trials, most notably differences in baseline vitamin D status, the vitamin D regimen, and the comparison group. For example, the 2013 systematic review by Jolliffe et al. included 14 trials, with discordant results [8]; approximately half reported benefits while half did not. Given this heterogeneity, the authors did not pool the trials for meta-analysis. Jolliffe et al. called for additional RCTs, particularly in populations with a high prevalence of vitamin D deficiency at baseline, using doses sufficient to raise 25(OH)D levels to potentially beneficial levels, and with sufficient statistical power to detect subgroup effects.

The 2013 systematic review by Bergman et al. included 11 trials ( $n = 5660$ ) which they did meta-analyze [159]. The authors found evidence of clinically and statistically significant benefit, with a summary OR of 0.64 (95%CI, 0.49–0.84). However, as the figure clearly shows, the RCT results are quite heterogeneous. The authors explored these differences and found, for example, that the protective effect against ARI was larger in trials that used once-daily dosing compared to bolus doses

(OR 0.51 vs. 0.86, respectively;  $P = .01$ ). More recently, in 2021, Abioye et al. pooled multiple micronutrient trials in adults and found a modest overall protective effect against ARI [161]. The authors found that the benefit was greater when the diagnosis was based on clinical diagnosis or laboratory testing, rather than self-report, and that the optimal dosing regimen would be a daily dose of at least 2000 IU, with a smaller loading dose (<60,000 IU). They also found that vitamin D supplementation shortened the duration of ARI symptoms mildly, though the effect did not differ by dose. Taken together, multiple systematic reviews have clarified that while vitamin D supplementation might indeed lower the risk of ARI, this benefit may apply only to specific individuals on specific vitamin D regimens; other individuals, on other regimens, may not derive any benefit at all.

To better study this effect modification, it would be helpful to have individual participant data (IPD) from every relevant RCT and to then analyze the newly compiled dataset in ways that are not possible in a traditional meta-analysis where the unit of observation is the entire trial. For example, if one were interested in the effect of vitamin D supplements on participants with low baseline levels of 25(OH)D, an IPD meta-analysis would permit the analyst to include every participant with a low baseline level of 25(OH)D, across all trials, into this one subgroup of interest. In 2017, Martineau et al. presented the first results of such a project [162]. Briefly, IPD from 25 RCTs worldwide were combined into a dataset with 10,933 participants. The overall finding was a protective effect against ARI (OR 0.88, 95%CI 0.81–0.96), with marked heterogeneity across the trials ( $P < .001$ ). Prespecified subgroup analysis revealed that daily-or-weekly dosing (without bolus) provided benefit (adjusted OR 0.81; 95%CI, 0.72–0.91) while those receiving one or more bolus doses did not (adjusted OR 0.97; 95%CI, 0.86–1.10;  $P$  for interaction = 0.05). Among those receiving daily or weekly vitamin D, protective effects were stronger among those with baseline 25(OH)D < 10 ng/mL (adjusted OR 0.30; 95%CI, 0.17–0.53) compared to those with baseline levels  $\geq 10$  ng/mL (adjusted OR 0.75; 95%CI, 0.60–0.95;  $P$  for interaction = 0.006).

In 2021, the IPD meta-analysis was updated with more RCTs, including some that had been started long before the 2017 publication [163]. For example, a large trial from New Zealand compared bolus dosing (200,000 IU at baseline, then monthly 100,000 IU) versus placebo and, consistent with the 2017 conclusions, the bolus dosing regimen showed no ARI benefit [164]. Adding more bolus trials to the IPD meta-analysis will, of course, weaken the overall “composite” finding but it should have little impact on the finding that daily dosing appears beneficial. Indeed, the 2021 IPD meta-analysis, with 43 eligible trials ( $n = 48,488$ ), showed

that a slightly lower proportion of participants in the vitamin D supplement group had one or more ARIs than in the placebo group (OR 0.92; 95%CI, 0.86–0.99). Again, there was evidence of significant heterogeneity across the trials ( $P = .018$ ).

Of note, the 2021 IPD meta-analysis also added a large trial from Mongolia that compared weekly doses of 14,000 IU versus placebo in Mongolian children with low vitamin D status and, surprisingly, it found no significant ARI benefit [71]. While the explanation for the Mongolia trial results is unclear, one possibility is that the children were effectively receiving bolus doses (albeit weekly) given their young age and size. Perhaps they would have benefitted from a smaller daily dosing? Regardless, the inclusion of this large number of children with vitamin D deficiency contributed to the loss of baseline 25(OH)D level as a predictor of vitamin D supplement benefit [163].

The 2021 IPD meta-analysis affirmed the value (all ORs  $<0.80$ ) of daily dosing, at a daily dose equivalent of 400–1000 IU, for a duration of 12 months or less [163]. There also was evidence of enhanced benefit among younger participants (ages 1–15.9 years). The authors concluded that despite evidence of significant heterogeneity across trials, vitamin D supplementation was safe and reduced the risk of ARI compared with placebo. While the overall effect was modest (OR 0.92), the benefit among a subgroup of the enrolled participants receiving daily dosing was substantial.

Taken together, RCTs to date, while not uniform in their results, suggest that vitamin D supplementation really can lower the risk of ARI, particularly in those at low baseline 25(OH)D levels and when the supplement is taken daily without bolus. As more RCT results emerge in the literature, we hope to add the IPD of these trials to the ongoing IPD meta-analysis to further refine our understanding. Based on the trials to date, adding more bolus trials will push the overall composite finding (any vitamin D supplement) toward the null. One hopes, however, that trialists will be guided by a more “precision medicine” approach and that they will design trials that are the most likely to show benefit.

## 5. Vitamin D and asthma

### 5.1 Asthma pathogenesis

Given the early age of asthma onset [50,51], and growing evidence for the developmental origins of obstructive lung diseases [52,165], the potential role of maternal diet during pregnancy on the asthma risk of offspring continues to be an active area of research worldwide. However, while investigators have debated the possible role of micronutrient intake on asthma risk

for many decades, multiple reviews of diet and asthma—from 1997 to 2005—said nothing about vitamin D as a potential risk factor for asthma [80–82,166].

As discussed by Camargo et al. in their 2007 publication [77], epidemiologic data in the mid-2000s suggested a possible association between vitamin D deficiency and the asthma epidemic. For example, the prevalence of both conditions is higher in racial/ethnic minorities, obese individuals, and westernized populations. Moreover, a large cross-sectional study in 2005 had shown that vitamin D deficiency was correlated with lower pulmonary function, including forced expiratory volume in 1 s (FEV<sub>1</sub>) and forced vital capacity [83]. Of course, the causality of this cross-sectional association was uncertain; although low vitamin D status might cause asthma (or lower pulmonary function), it also was possible that individuals with asthma were less likely to exercise outdoors and that this and other lifestyle differences could have caused the vitamin D deficiency. Similarly, lingering concerns about the adverse effect of milk on asthma may have led some asthmatic individuals to avoid milk intake (a major dietary source of vitamin D in US children [77]), and this food avoidance also could create a spurious inverse association between vitamin D intake and asthma. Prospective studies (especially RCTs) would help to address these major methodological concerns. These studies would be challenging, of course, given the duration of follow-up required—e.g., until at least 5–6 years of age for a diagnosis of childhood asthma [50] and until late adolescence for lung development [84].

As noted earlier, the hypothesis that vitamin D deficiency may cause asthma—and that vitamin D supplements may prevent asthma—was disputed by Wjst, who repeatedly described vitamin D supplementation as an important *cause* of asthma [9,85,86]. The strongest support for this putative harm came from a large birth cohort in northern Finland [87]. In this 2004 publication, Hypponen, Wjst et al. reported that regular vitamin D supplementation ( $\geq 2000$  IU/d) in the first year of life increased the risks of developing atopy (OR 1.46), allergic rhinitis (OR 1.66), and asthma (OR 1.35), by age 31 years. For reasons that will be discussed later in this chapter, this finding may be related to the very high dose of vitamin D supplementation used in these infants (i.e., this may represent a dose-specific effect during a critical window). Regardless, this retrospective cohort study also was limited by the absence of data on maternal intake of vitamin D and the inability to control for major confounders. Furthermore, recall bias may have affected the ascertainment of early-life asthma and allergies.

Nevertheless, other contemporaneous European publications also have suggested that vitamin D supplements may increase asthma/allergy risk [88,89]. Gale

et al. studied child health outcomes in a UK birth cohort and found that higher levels of maternal 25(OH)D (measured at a median of 33 weeks gestation) were associated with an increased risk of atopic outcomes [88]. More specifically, children whose mothers had 25(OH)D levels in pregnancy  $>30$  ng/mL had an increased risk of eczema at 9 months (OR 3.26) and asthma at 9 years (OR 5.40), compared to children whose mothers had levels  $<12$  ng/mL. Unfortunately, this study also has several methodological limitations, including small numbers of outcomes and poor follow-up (e.g., only 30% at age 9 years); the authors appropriately called for confirmation of these associations in other studies. The UK findings were similar, however, to those of Back et al. in Sweden [89]. In this small cohort of 123 children, the investigators found higher age 6-year prevalence of atopic conditions among children who had taken vitamin D supplements during infancy. In another northern European study, Hansen et al. in Denmark, investigators reported that high maternal 25(OH)D levels during pregnancy (i.e.,  $\geq 50$  ng/mL) may have been associated with offspring having a higher risk of asthma outcomes during adulthood, as compared to children of women with maternal levels of  $<20$  ng/mL [90]. More recently, Nwaru et al. in Finland reported that increased vitamin D intake (particularly at ages 1 and 2 years), may increase the risk of childhood asthma [91]. In sum, although all of these European studies acknowledge methodological problems, the combined data do raise important potential safety concerns about high-dose vitamin D supplementation in late pregnancy or infancy.

Concerns about vitamin D in general are mitigated by several epidemiologic studies showing that vitamin D has no apparent association with the development of true childhood asthma. As described in the section on "Vitamin D and ARI," birth cohorts in both Boston [77] and Scotland [79] found an inverse association between maternal intake of vitamin D during pregnancy and risk of recurrent childhood wheezing, which is not synonymous with true asthma. The Scottish investigators reported that lower vitamin D intake during pregnancy was associated with a borderline significant decrease in bronchodilator response ( $P = .04$ ), but there was no association with doctor-diagnosed asthma or other asthma-related outcomes, such as spirometry or exhaled nitric oxide concentration [79]. Both studies were limited, however, by their estimation of vitamin D status from oral intake only. Thus, the unique contribution of the New Zealand birth cohort [146] was the concurrent demonstration that low cord blood 25(OH)D levels were associated with an increased risk of ARIs and childhood wheezing, but had no association with the risk of current asthma at age 5 years (Fig. 105.2). The lack of association between vitamin D and incident

asthma was present for both atopic asthma and non-atopic asthma, with atopy defined by skin-prick testing for common allergens at age 15 months.

Since the publication of these three birth cohort studies [77,79,146], many birth cohorts from around the world have examined these same questions, using different measures of vitamin D status and different definitions of "asthma" (ranging from recurrent wheezing during early childhood to an actual confirmed diagnosis by a physician). As previously discussed, two systematic reviews did not find an association between maternal intake of vitamin D during pregnancy and the risk of offspring asthma [143,145]. Based on these null findings, and notwithstanding the compelling rat model data on lung development (chapter Section 3), there is little epidemiologic support for vitamin D supplementation during pregnancy as an effective strategy for the primary prevention of childhood asthma.

While this discussion has focused heavily on childhood asthma, researchers over the past few years have started to study a possible link between adult vitamin D status and adult-onset asthma. For example, Mai et al. reported evidence from a large population-based cohort in Norway and found that vitamin D status, as measured by serum 25(OH)D level, was unrelated to adult-onset asthma in most adults [92]. On the contrary, these investigators found that a higher intake of cod liver oil, a traditional source of vitamins A and D in Nordic countries, was associated with an increased risk of developing adult-onset asthma [93]; the authors speculated that this excess risk may have been due to the high vitamin A content of the cod liver oil preparation.

As noted earlier, the link between vitamin D and asthma also has been examined using genetic data. Although preliminary evidence from two family-based studies suggested that specific VDR gene polymorphisms were associated with asthma among North American children and adults [10,11], the results were inconsistent across cohorts/studies and were not confirmed [12,13]. A 2009 study by Bosse et al. reported that several genes involved in the vitamin D pathway (e.g., *IL10*, *CYP24A1*, *VDR*) had "modest levels of association" with asthma and atopy but the specific SNPs, or the orientation of the risk alleles, were different between populations [94]. In a 2016 systematic review by Han et al., the authors identified nine eligible studies (2116 asthma patients and 1884 healthy controls) that examined the association between VDR polymorphisms and asthma risk [95]. The meta-analysis showed that rs2228570, rs7975232, rs731236 and rs3782905 gene polymorphisms in VDR were associated with increased susceptibility to asthma. In another systematic review, Zhao et al. also focused on VDR polymorphisms but stratified their meta-analysis by ethnicity [96]. These authors



report significant associations for FokI (rs2228570) among whites, and ApaI (rs7975232) among Asians; other gene polymorphisms were not significantly associated. With regard to *DBP* (GC) polymorphisms, a paper by Navas-Nazario et al. found that, compared with the wild-type genotype DT/DT (Gc1f/Gc1f), the ET/ET (Gc1s/Gc1s) genotype of *DBP* was less associated with childhood asthma [97]. Recently, in 2020, Makoui et al. reported a meta-analysis, based on 17 case-control studies, with a goal of identifying whether *VDR* gene polymorphisms play a role in risk of asthma [98]. The results of the pooled analysis revealed significant “protective” associations between FokI SNP and TaqI polymorphisms with asthma risk. Moreover, subgroup analyses showed race/ethnicity influences asthma risk in Asian, African, and American populations.

The implications of these genetic findings for the primary prevention of asthma remain unclear and require further investigation. *VDR* polymorphisms are discussed further in Chapters 60 and 61 and *DBP* polymorphisms in Chapters 7 and 60. On a final genetic tangent, Hysinger et al. reported an MR analysis that found that low vitamin D status is unlikely to be causative for childhood asthma [99].

Taking all of this evidence under consideration, it seems unlikely that vitamin D deficiency during pregnancy (or early infancy) is a major contributor to the current global asthma epidemic. Nevertheless, if a modest association exists between vitamin D deficiency and incident asthma, it might be mediated through increased risk of specific types of ARIs, or perhaps a high frequency of ARIs, in early life. In a 2008 paper from the Childhood Origins of Asthma (COAST) birth cohort in Wisconsin, Jackson et al. [100] reported that wheezing rhinovirus illness during infancy strongly predicted the development of asthma at age 6 years. A 2019 study in a large cohort of children with a history of severe bronchiolitis confirmed that RV bronchiolitis conveys increased risk when combined with atopy [101]. Given the connection between vitamin D deficiency and ARI risk (see earlier section), and inconsistent laboratory data on the effect of vitamin D on rhinovirus replication [102,103], it remains possible that “healthy” levels of vitamin D might prevent some cases of rhinovirus ARI and thereby lower future risk of incident asthma. Unfortunately, the COAST study also was the birth cohort where cord blood 25(OH)D levels were unrelated to either childhood wheezing or age 6-year asthma [148]; repeat 25(OH)D levels later in childhood also yielded nonsignificant associations in this Wisconsin cohort (Camargo et al., unpublished data). While the vitamin D-ARI-asthma hypothesis merits further investigation, it does not look promising. Moreover, this speculation must be balanced against several studies suggesting that excessive vitamin D supplementation during pregnancy and infancy may increase

the risk of asthma and allergies in some children, either during childhood or early adulthood [87–90].

While this evidence base was building, several large RCTs were launched to better examine the hypothesis that prenatal vitamin D supplementation might prevent childhood asthma. A 2016 systematic review by Vahdaninia et al. [104] identified three eligible RCTs on this topic, with the important caveat that all three trials used *childhood wheezing by age 3 years* as a proxy for asthma. Pooled analysis of the 1387 participants in the two major trials—one by Chawes et al. in Denmark, which compared 2400 IU/day versus placebo [105], and the other by Litonjua et al. in the United States, which compared 4000 IU/day versus placebo [106]—showed that daily vitamin D supplementation during pregnancy significantly reduced risk of childhood wheezing (RR 0.80; 95%CI, 0.66–0.97). The inclusion of the smaller RCT by Goldring et al. [107], which involved a more complex study design, did not materially affect the results (RR 0.82; 95%CI, 0.68–0.99). The authors of the systematic review concluded that daily vitamin D supplementation during pregnancy appears to protect against the development of childhood wheezing but cautioned that “early childhood wheeze is not the same as asthma.” They called for longer-term follow-up of these RCT cohorts to establish the efficacy of prenatal vitamin D supplementation in the primary prevention of actual asthma.

Indeed, with the passage of time, both the Danish and USA trials have reported on the impact of vitamin D supplementation during pregnancy on the risk of asthma at age 6 years. Neither trial found an association between the prenatal intervention and asthma risk [108,109]. The consistency of these findings is striking, with evidence of benefit in early life (presumably ARI-related wheezing) but no apparent impact on actual childhood asthma. Recently, Kihltila et al. reported a novel twist on the recurrent wheezing results [110]. Briefly, they reported that maternal 17q21 genotype had an important influence on the protective effect of prenatal vitamin D supplementation on offspring recurrent wheeze in both trials, with significantly reduced risk among mothers with low-risk GG or GC genotype but no such difference among offspring of mothers with the high-risk CC genotype. This novel finding merits further investigation.

Finally, although a fourth RCT did not meet the eligibility criteria of the Vahdaninia systematic review, this work from New Zealand provides additional insights [111]. Briefly, Grant et al. enrolled 266 pregnant women who were randomized to three groups: placebo, lower-dose vitamin D (1000 IU daily), and higher-dose vitamin D (2000 IU daily). Unlike the other three RCTs, which stopped the vitamin D intervention at birth [106,107] or at 1 week postpartum [105], the New Zealand trial

continued the vitamin D intervention in the infants for 6 months postpartum. In correspondence to the randomly assigned group of the mother, infants were assigned to placebo, 400 IU/day, and 800 IU/day. In a post hoc analysis of primary care visits from birth until age 18 months, the higher-dose vitamin D group experienced significantly less total ARI visits per child than those in the placebo group. Although reported in this section of the chapter—because of its pregnancy/infancy design—this New Zealand RCT focused on ARI; these data contributed to the previously discussed IPD meta-analyses on vitamin D supplementation and risk of ARI [162,163].

## 5.2 Asthma control, including exacerbations

Although improved vitamin D status does not appear to affect the risk of incident asthma, it could have an important role in asthma management among those who already have developed the disease. In other words, vitamin D may have little role in disease prevention (primary prevention) but an important role in disease modification (secondary prevention). The potential benefits are based on two interrelated concepts: (1) responsiveness to corticosteroids and (2) risk of exacerbations.

### 5.2.1 Responsiveness to corticosteroids

With regard to corticosteroids, most individuals with asthma respond well to inhaled corticosteroids, with demonstrated reductions in symptoms and serious exacerbations and improved pulmonary function and asthma-related quality of life [43,44]. For these reasons, asthma guidelines uniformly consider inhaled corticosteroids the preferred long-term control medication for asthma. Nevertheless, asthma control on even “optimal” controller therapy often leaves room for improvement [112]. This is particularly true in a relatively small subset of asthma patients with corticosteroid resistance [113]. In 2006, Xystrakis et al. reported the effect of calcitriol treatment in this asthma subgroup [114]. The investigators administered oral 1,25(OH)<sub>2</sub>D (0.5 µg/day for 7 days) to a small group of healthy individuals and corticosteroid-resistant asthma patients and found that the intervention enhanced subsequent responsiveness to dexamethasone due to induction of interleukin (IL)-10. The investigators concluded that vitamin D could potentially increase the therapeutic response to corticosteroids in otherwise resistant asthma patients.

Over the past 15 years, several groups have built on this innovative work. In 2010, for example, Sutherland et al. studied 54 adults with asthma to determine the relation of serum 25(OH)D levels to asthma phenotype and corticosteroid response [115]. In this cross-

sectional study, patients with lower serum 25(OH)D levels were more likely to have impaired lung function and increased airway hyper-responsiveness. Among the subset of patients not on inhaled corticosteroids, lower 25(OH)D levels were correlated with lower corticosteroid response (as measured by dexamethasone-induced expression of mitogen-activated protein kinase phosphatase (MPK)-1 by peripheral blood cells); this finding was not seen among patients on inhaled corticosteroids. Searing et al. studied similar issues in 100 asthmatic children [116]. In the cross-sectional part of their study, lower serum 25(OH)D levels were associated with greater corticosteroid use and worse airflow limitation. Moreover, the investigators found the amount of MPK-1 and IL10 mRNA induced by dexamethasone plus 1,25(OH)<sub>2</sub>D was significantly greater than that induced by dexamethasone alone ( $P < .01$ ). In an experimental model of corticosteroid resistance in which dexamethasone alone did not inhibit T-cell proliferation, the addition of 1,25(OH)<sub>2</sub>D caused significant dose-dependent suppression of cell proliferation. In 2012, Goleva et al. extended this research by demonstrating significant associations between serum 25(OH)D levels and corticosteroid requirement and in vitro responsiveness to corticosteroids in asthmatic children but not asthmatic adults [117]; likewise, 25(OH)D was associated with IgE in children but not in adults. In 2015, Chambers et al. identified both IL-17A<sup>high</sup> and IFN-γ<sup>high</sup> immunophenotypes in patients with corticosteroid-resistant asthma and showed that calcitriol potentially improves the clinical response to corticosteroids through reduction of IL-18A production and enhancement of corticosteroid-induced IL-10 [118]. The authors suggest that these laboratory findings might help to identify vitamin D responder immunophenotypes. Recently, Jiang et al. used a proteomic approach to identify biomarkers in a small sample and found that increased DBP might be a useful biomarker for predicting corticosteroid-resistance in asthma patients [119]. While this final study was quite small and exploratory, the authors' convergence on DBP provides another interesting link between vitamin D and corticosteroids in asthma.

In the epidemiologic realm, several studies have identified a link between vitamin D status and inhaled corticosteroid requirements and effectiveness. For example, in 2012, Wu et al. reported the association between baseline vitamin D status and the effect of inhaled corticosteroid treatment on lung function in children [120]. The investigators analyzed data from 1024 asthmatic children in the Childhood Asthma Management Program (CAMP) where 10% had baseline 25(OH)D levels <20 ng/mL, 25% were 20–30 ng/mL, and 65% were >30 ng/mL. In the inhaled corticosteroid treatment group, prebronchodilator FEV1 increased from randomization to 12 months by 140 mL in the <20 ng/

mL group, by 330 mL in the 20–30 ng/mL group, and by 290 mL in the >30 ng/mL group ( $P = .007$ ). The authors recommended monitoring 25(OH)D levels when treating children with persistent asthma with inhaled corticosteroids.

In this context, in 2014, Castro et al. reported RCT results on the effect of vitamin D supplementation in adults with symptomatic asthma and baseline 25(OH)D  $< 30$  ng/mL [121]. The 408 subjects were randomly assigned to either vitamin D (100,000 IU bolus, then 4000 IU/day for 28 weeks,  $n = 201$ ) or placebo ( $n = 208$ ), with both groups also receiving an inhaled corticosteroid, ciclesonide 320  $\mu$ g daily. If asthma control was achieved after 12 weeks, ciclesonide was tapered per protocol. The primary outcome (asthma treatment failure) will be addressed later in this section (along with other studies of vitamin D and asthma exacerbation) but one of the secondary outcomes is relevant here. Briefly, the RCT revealed that the average overall dose of the inhaled corticosteroid required to maintain asthma control was lower in the vitamin D group than in the placebo group. While the average  $\mu$ g difference was admittedly small, one wonders what the RCT results might have looked like in adults (or children) with lower baseline 25(OH)D levels and higher corticosteroid resistance? Moreover, building on the emerging evidence against the bolus dosing for ARI prevention, what impact did the initial 100,000 IU bolus have on these study results? Regardless, the reported finding is consistent with a growing body of evidence that supports a clinically significant hormonal interaction between corticosteroids and vitamin D in at least some patients with asthma.

### 5.2.2 Asthma exacerbations

With regard to asthma exacerbations, it is important to remember that viral ARIs, particularly human rhinovirus, are associated with most exacerbations [48,49]. While adults typically experience 2 to 4 upper ARIs per year, young children experience 6 to 10 per year [41]. Furthermore, individuals with asthma may be more susceptible to ARI and have an increased frequency of lower respiratory tract symptoms of higher severity and duration [122]. As described in more detail in Chapters 94–99, the role of vitamin D in innate immune responses may explain a predisposition to ARI and asthma exacerbation in vitamin D deficient populations.

As discussed earlier in this chapter, there is substantial evidence of a “protective” association between vitamin D and ARI in a subset of the general population, with RCT evidence suggesting the specific individuals (e.g., low baseline 25(OH)D levels, children) and vitamin D regimens (e.g., daily dosing without any bolus) that are most likely to demonstrate benefit. To date, however,

there are substantially less data on this vitamin D-ARI link among asthmatic individuals. In a 2009 publication based on nationally-representative data from NHANES-III [7], the association between serum 25(OH)D levels ( $<10$  ng/mL versus  $\geq 30$  ng/mL) and upper ARI was much stronger among individuals with asthma (OR 5.67) compared to those without asthma (OR 1.24;  $P$  for interaction = 0.007). Consistent findings were reported from a cross-sectional study by Brehm et al. on 616 Costa Rican children [123]. In multivariable models, lower serum 25(OH)D levels were associated with higher total IgE and eosinophil count, increased airway responsiveness, higher likelihood to have been hospitalized for asthma in the past year, and a higher likelihood of having used an antiinflammatory medication in the past year. Although these findings sound compelling, the cross-sectional design leaves room for reverse-causality. While upper ARIs are unlikely to cause a lasting reduction in serum 25(OH)D level, one might reasonably ask if children with severe persistent asthma are more likely to stay indoors—and, as a result, have lower serum 25(OH)D levels?

To that end, Brehm et al. examined the *longitudinal* association between serum 25(OH)D levels and the risk of severe asthma exacerbations in the CAMP study [124]. Children with low baseline 25(OH)D levels ( $<30$  ng/mL) were more likely to have a severe asthma exacerbation over a 4-month follow-up period (OR 1.5; 95%CI 1.1–1.9). As noted earlier, the original CAMP trial involved randomization to different asthma treatments and the investigators reported a “protective” association between 25(OH)D level and severe exacerbation in children randomized to budesonide (OR 1.8; 95% 1.0–3.2), as well as those randomized to either placebo or nedocromil (OR 1.3; 95%CI, 1.0–1.9). These observational data support the potential role of vitamin D as an adjunct treatment for asthmatic children with low baseline 25(OH)D levels.

Over the past decade, epidemiology research on vitamin D and asthma management continues to support these general observations but it also has expanded to other areas of asthma control. For example, Brumpton et al. examined the association between 25(OH)D levels and lung function decline in 395 Norwegian adults with asthma [125]. The investigators found that low 25(OH)D levels ( $<20$  ng/mL) were weakly associated with more lung function decline in the entire sample, but that there were stronger associations among specific asthma subgroups (i.e., never smokers and those not using inhaled corticosteroids). In a subsequent paper, looking at 25(OH)D level, vitamin D supplement, and asthma control [126], the authors reported little evidence that, among adults with asthma, having a low 25(OH)D level of being a nonuser of vitamin D supplement was associated with poor asthma control. While these cohort

findings require replication, they are cited to demonstrate some of the newer areas of vitamin D-asthma research.

A more conventional approach to improving asthma control is to reduce exacerbations and, for that, there now are several RCTs on supplementation with vitamin D versus placebo among asthmatic individuals of all ages. For example, in 2015, Martineau et al. reported an RCT where 250 adults were given six 2-monthly doses of 120,000 IU of vitamin D or placebo over 1 year [127]. The intervention did not influence time to first severe exacerbation or first upper ARI; indeed, the investigators did not find any clinically important effect of vitamin D for any secondary outcome. Moreover, these results were not modified by baseline 25(OH)D level or genotype.

The previously introduced RCT by Castro et al. also addressed the impact of vitamin D on the clinical course of adults with asthma [121]. In this 2014 publication, the primary outcome was first “asthma treatment failure”, which was a composite outcome of decline in lung function and increases in use of beta-agonists, systemic corticosteroids, and health care. As the primary outcome, the authors focused on this result which was completely null (adjusted hazard ratio 0.9, 95%CI 0.6–1.3,  $P = .54$ ). However, the investigators also reported an outcome of greater relevance to this discussion, the number of asthma exacerbations over the entire 28-week study period. For that outcome, the vitamin D group had 0.26 events per person-year while the placebo group had 0.40 (adjusted hazard ratio 0.63, 95%CI 0.39–1.01,  $P = .05$ ). Thus, despite the initial 100,000 IU bolus and enrollment of a relatively broad panel of adults, this reportedly “null” RCT provided evidence that daily vitamin D supplementation may lower the risk of exacerbations in at least some adults with asthma. In a recently published post hoc analysis by the investigators [128], Jiao et al. report statistically significant decreases in asthma exacerbation event rates among subjects without sinonasal disease (adjusted RR 0.3, 95%CI 0.1–0.7,  $P = .01$ ) but warned of a possibly higher rate of exacerbation among black asthmatic adults with sinonasal disease (adjusted RR 2.0, 95%CI 0.7–5.5,  $P = .17$ ). The inclusion of these and other data into our ongoing IPD meta-analyses [162,163] could help to determine if the nonsignificant signal among blacks is a chance finding or of actual concern.

In a 2015 systematic review on RCTs of vitamin D supplementation and ARI in children, Xiao et al. reported favorable results for asthma exacerbation [129]. They included seven eligible trials and provided a summary estimate for ARI that, while not statistically significant (RR 0.79, 95%CI 0.55–1.13), had a point estimate comparable to that in prior studies. More importantly, the pooled data from the two trials with asthma data

[130,131], which both used daily dosing, showed a dramatic reduction in exacerbation risk (RR 0.26; 95%CI, 0.11–0.59). The authors concluded that their findings did not support the routine use of vitamin D supplementation for ARI prevention in healthy children but that supplementation was of likely benefit among children with asthma. In view of the IPD meta-analysis data regarding ARI [162,163], it also seems reasonable to surmise that vitamin D supplementation, when used, should avoid bolus dosing.

In 2017, Jolliffe et al. published an IPD meta-analysis of RCTs that examined the effect of vitamin D supplementation on the risk of asthma exacerbations [132]. Briefly, eight RCTs were included, with a total of 1078 participants. Vitamin D supplementation reduced the rate of asthma exacerbation requiring treatment with systemic corticosteroids among all participants (adjusted IRR 0.74; 95%CI, 0.56–0.97). Subgroup analyses suggested that protective effects may have been larger in participants with baseline 25(OH)D < 10 ng/mL (adjusted IRR 0.33; 95%CI, 0.11–0.98) than among those with higher levels (adjusted IRR 0.77; 95%CI, 0.58–1.03) but the formal test for interaction was not significant ( $P$  for interaction = 0.25). Although statistical power was limited, the IPD meta-analysis did not suggest that daily versus bolus dosing mattered.

In 2020, Forno et al. published an RCT looking at whether vitamin D supplementation reduces the risk of severe childhood asthma exacerbations [133]. Briefly, participants were randomized to 4000 IU/day or placebo and there was no difference in exacerbation rates. A secondary outcome was the effect of the supplement on ICS dose and no differences were observed. The participants were described as having “low vitamin D levels” (i.e., <30 ng/mL), with baseline values of approximately 23 ng/mL; participants with truly low levels were excluded.

In 2021, Jat et al. reported another RCT of vitamin D supplementation in children with asthma [134]. The 250 Indian children received 1000 IU daily for 9 months or a similar-looking placebo and, again, there was no evidence of improved asthma control. Baseline 25(OH)D levels were <15 ng/mL in this trial, with a rise to 18 ng/mL in the intervention group. The explanation for the discordant results is not yet clear. It will be helpful to add these trial data to the ongoing IPD meta-analyses to pursue potential effect modifiers.

## 6. Vitamin D and Copd

### 6.1 COPD pathogenesis and progression

While many studies have looked at the potential role of vitamin D in the etiology of asthma, there are much



less data on whether vitamin D is associated with the risk of developing COPD. An early (2013) review article by Janssens et al. listed several biologically-plausible mechanisms by which low vitamin D status might contribute to COPD, and summarized studies showing that COPD patients tend to have low vitamin D status [135]. They acknowledged the nonetiologic explanations for this cross-sectional finding (e.g., older individuals with COPD may simply spend less time outdoors or eat differently than their healthier, non-smoking counterparts). While cohort studies of 25(OH)D status and lung function could better address this reverse causality, they still may be subject to significant confounding by smoking and exercise [136], which have their own complex associations with other lifestyle factors and diseases. Moreover, smoking status might modify the association between vitamin D and lung health, or, stated differently, vitamin D might offset some of the adverse effects of smoking on the lung [137,138]. In light of the relatively sparse data, the 2013 review called for large population-based and clinical cohort studies to investigate whether vitamin D status influences the risk of developing COPD, decline in lung function, or AECOPD frequency. (AECOPD will be addressed in the next section).

In 2014, Afzal et al. published the first population-based study on vitamin D status and risk of COPD [139]. Briefly, plasma 25(OH)D levels were measured in the Copenhagen City Heart Study ( $n = 10,116$ ) and the Copenhagen General Population Study ( $n = 8391$ ); in the former study, up to three spirometry measurements were taken during approximately 20 years of follow-up, which permitted analysis of lung function decline. The investigators found that low 25(OH)D levels were associated with faster lung function decline and lower lung function. Moreover, low baseline 25(OH)D levels were associated with a higher risk of incident COPD. These Danish results are striking but await replication in other large prospective cohorts. For example, in the Nurses' Health Study ( $n = 64,494$  women) and Health Professionals Follow-up Study ( $n = 45,421$  men), we have not found any association between vitamin D status and risk of incident COPD (Camargo et al., unpublished data).

In the genetic realm, several studies over the past 25 years have suggested a possible association between DBP polymorphisms and COPD. In 2015, this topic was the focus of several meta-analyses from China. For example, Xiao et al. identified seven eligible studies ( $n = 1475$ ) and found that GC1f was a risk factor for COPD in Asians, whereas GC1f was a protective factor against COPD in whites [140]. Chen et al. identified eight eligible studies ( $n = 2216$  patients) and found, among Asian patients only, that the GC1f was a risk factor, while the GC1S and GC2 were protective [141]. Xie

et al. identified 12 studies ( $n = 2937$ ) and found, among Asians only, that GC1f was a risk factor, while GC2 was protective [142]. Clearly, more data are needed to clarify these disparate findings, with particular attention to the role of race/ethnicity. DBP polymorphism data are discussed further in Chapters 7 and 60.

To address the hypothesis that low vitamin D status might increase lung function decline among patients with prevalent COPD, Kunisaki et al. examined serum 25(OH)D levels and *longitudinal* lung function decline in the Lung Health Study three cohort [167]. The investigators performed a nested (prospective) case-control study involving 196 COPD patients with either rapid (case) or slow (control) decline in FEV<sub>1</sub> over approximately 6 years of follow-up. Despite rapid and slow decliners experiencing strikingly different rates of FEV<sub>1</sub> decline ( $-151$  vs.  $0$  mL/year,  $P < .001$ ), there was no significant difference in baseline 25(OH)D levels between the two groups ( $25.0$  vs.  $25.9$  ng/mL,  $P = .54$ ). While these findings require replication, they suggest that vitamin D status is not associated with lung function decline among patients with COPD. By contrast, a 2015 study by Persson et al. in Norway followed 426 COPD patients every 6 months for several years [78]. The one-third of the patients with baseline 25(OH)D  $< 20$  ng/mL tended to experience a greater decline in both FEV<sub>1</sub> and FVC, compared to patients with higher levels; for FEV<sub>1</sub>, this difference was statistically significant only for the subset of COPD patients with baseline 25(OH)D levels  $< 10$  ng/mL.

In a relevant study, Sluyter et al. examined the effect of monthly vitamin D supplementation on lung function in a large New Zealand RCT of older adults [168]. Briefly, the intervention group received 100,000 IU monthly, while the comparison group received placebo. There were no significant lung function improvements in the total sample, vitamin D deficient participants, or those with asthma/COPD. However, among ever smokers ( $n = 217$ ), the mean (95%CI) FEV<sub>1</sub> increase in the vitamin D versus placebo was 57 [4–77], [79–86], [143–166] mL ( $P = .04$ ). FEV<sub>1</sub> improvements were largest among vitamin D deficient ever-smokers with asthma COPD ( $n = 60$ ): 160 (53, 268); ( $P = .004$ ). Thus, vitamin D supplementation did not benefit everyone but benefitted ever-smokers, especially those with vitamin D deficiency or asthma/COPD.

In summary, while many COPD patients have vitamin D deficiency [135] and may warrant vitamin D supplementation for general health reasons, the role of vitamin D supplementation in the primary prevention of COPD awaits more robust data from additional prospective cohort studies and, if warranted, future RCTs. Likewise, the role of vitamin D supplementation on the progression of lung function decline in COPD patients requires further study.

## 6.2 Acute exacerbations of COPD

Similar to asthma exacerbations, most AECOPDs are caused by common respiratory viruses [57]. Since vitamin D-mediated immune mechanisms appear to play a role in the prevention of viral ARI, vitamin D supplementation might be helpful against AECOPD. However, AECOPD is a more complicated phenomenon than asthma exacerbation. For starters, AECOPD often involve both viruses and bacteria [57]. Moreover, the typical COPD patient is older and more likely to suffer from other relevant smoking-related comorbidities, such as cardiovascular disease [169]. Nevertheless, the question remains: does vitamin D supplementation lower the risk of AECOPD?

In 2012, Kunisaki et al. analyzed data from an azithromycin trial of exacerbation-prone patients with COPD to examine the longitudinal association between baseline 25(OH)D levels and the risk of AECOPD over 1 year [170]. Contrary to their hypothesis, baseline 25(OH)D levels were unrelated to the risk of AECOPD in their observational study of this high-risk population. Similarly, null findings were reported in a 2014 study by Puhan et al. from the Netherlands and Switzerland [171] and in the previously cited 2015 study by Persson et al. in Norway [78]. Taken together, these observational studies do not support the role of vitamin D supplementation in the prevention of AECOPD.

In 2012, however, Lehouck et al. published the first major RCT on the effect of vitamin D supplementation on the risk of AECOPD [172]. This Belgian trial involved 182 patients with moderate to very severe COPD and a history of recent exacerbations; subjects were randomly assigned to a monthly dose of vitamin D (100,000 IU) or placebo for 1 year. The primary outcome was time to first AECOPD and the results were null (hazard ratio 1.1, 95%CI 0.82–1.56,  $P = .41$ ), as were several secondary outcomes, including AECOPD rates, FEV<sub>1</sub>, and hospitalization. However, a post hoc analysis of the 30 patients with baseline 25(OH)D < 10 ng/mL showed a significant AECOPD reduction in the vitamin D group (RR 0.57, 95%CI 0.33–0.98,  $P = .04$ ). In a separate post hoc analysis of 50 participants, Hornikx et al. examined the potential effect of vitamin D intervention on pulmonary rehabilitation outcomes [173]. Compared to the placebo group, patients taking monthly boluses of vitamin D had significantly larger improvements in inspiratory muscle strength and maximal oxygen uptake, while quadriceps strength and 6-minute walking distance did not differ between groups. While not directly related to AECOPD, the rehabilitation findings, if replicated, suggest another way in which vitamin D supplementation might play a role in the long-term management of patients with COPD. For further discussion of the effects of vitamin D on muscle function, please see [Chapters 29 and 38](#).

In 2015, Martineau et al. reported the results of another important RCT in the vitamin D-AECOPD literature [174]. In this UK trial, 240 patients were given six 2-monthly doses of 120,000 IU of vitamin D or placebo over 1 year. Similar to the Lehouck trial [172], the bolus intervention did not influence time to first AECOPD or first upper ARI. However, also similar to the Lehouck trial, a prespecified subgroup analysis showed that vitamin D was protective in patients with baseline 25(OH)D < 20 ng/mL (hazard ratio 0.57, 95%CI 0.35–0.92,  $P = .02$ ) but not in those with higher baseline levels ( $P$  for interaction = 0.02). On the other hand, baseline 25(OH)D level did not modify the effect of the bolus vitamin D dosing on the risk of first upper ARI. The reason for the disconnect between ARI and AECOPD is not yet clear but the authors speculated, based on exploratory analyses, that vitamin D might have attenuated the potential for an upper ARI to precipitate AECOPD.

In 2019, Jolliffe et al. published an IPD meta-analysis of RCTs that examined the effect of vitamin D supplementation on the risk of AECOPD [175]. The analysis included only four RCTs ( $n = 560$ ), with IPD obtained from three RCTs ( $n = 469$ ). Although supplementation did not effect the overall rate of moderate/severe AECOPD (adjusted IRR 0.94; 95%CI, 0.78–1.13), a prespecified subgroup analysis revealed protective effects among participants with baseline 25(OH)D levels of < 10 ng/mL (adjusted IRR 0.55; 95%CI, 0.36–0.84) but not those with higher levels (adjusted IRR 1.04; 95%CI, 0.85–1.27;  $P$  for interaction = 0.015). Again, as with vitamin D for the prevention of asthma exacerbation, bolus dosing did not appear to matter.

Since the publication of this IPD meta-analysis, Rafiq et al. have published an important RCT on vitamin D [176]. They enrolled 155 COPD patients with baseline 25(OH)D levels < 20 ng/mL and allocated them to either vitamin D 16,800 IU weekly or placebo for 1 year. Briefly, the intervention did not reduce AECOPD. In a prespecified subgroup analysis of 31 participants with baseline levels between 6 and 10 ng/mL, no significant benefit was found (IRR 0.91; 95%, 0.43–1.93). This result differs from that of the three trials in the IPD meta-analysis and, once again, suggests the potential value of including these new data in the ongoing IPD meta-analysis to help understand potential effect modifiers.

On the other hand, Camargo et al. performed a post hoc analysis of data from a large RCT of older adults in New Zealand to examine the effect of monthly dosing on the risk of either asthma or COPD exacerbation [177]. Overall, the authors found that vitamin D supplementation did not affect exacerbation risk (HR 1.08; 95%CI, 0.84–1.39) but, among those with baseline 25(OH)D < 10 ng/mL, the HR was 0.11 (95%CI, 0.03–0.51;  $P$  for interaction = 0.001).

Taken together, the vitamin D-COPD literature is at an earlier stage of development than that on ARI, childhood wheezing, and asthma. Nevertheless, there are sufficient positive findings to warrant further study, particularly on the effect of vitamin D supplementation on AECOPD risk. While vitamin D will not help all patients with COPD, it seems likely that this safe and inexpensive intervention will help selected patients with COPD. Further studies are needed to try to identify this vitamin D-responsive patient group.

## 7. Potential mechanisms

In addition to the likely role of vitamin D in lung development (see [Section 4](#) of this chapter), vitamin D also is known to have myriad effects on the immune system. Briefly, there now is substantial evidence that vitamin D has important roles in both the innate and adaptive immune systems. This enormous topic is covered in detail in other dedicated parts of [Section 11](#) (Chapters 94–96).

Moreover, vitamin D appears to influence the development of several atopic/allergic diseases, including atopic dermatitis, food allergy, and allergic rhinitis [178,179]. Given the extraordinary growth of research in these topics, interested readers are referred to the cited articles for further details. Briefly, however, there are studies to suggest that vitamin D deficiency during pregnancy or infancy increases the risk of each of these disorders, while other studies suggest that higher vitamin D status confers increased risk—or that there is no association at all! To date, few RCT results are available to test if vitamin D supplementation can prevent any of these disorders, but emerging trials suggest that vitamin D supplementation may help to prevent sensitization to common allergens [180] and this would presumably interrupt the pathway to actual allergic disease. Likewise, for specific patient subgroups, there already are RCT results that support vitamin D supplementation for disease modification (e.g., vitamin D for winter-related AD [181,182]). Along those lines, the 2006 RCT by Byremo et al. deserves mention [183]. Although the investigators did not mention vitamin D in their article, they demonstrated that moving from Norway to sunny Gran Canary appeared to benefit patients with severe AD, including significant decreases in bacterial skin colonization with *S. aureus*. Whether sunlight has distinct effects, beyond vitamin D synthesis, is another promising topic for future research.

Clearly, there is a complex connection between vitamin D and the different atopy/allergy disorders and attempts to describe the vitamin D hormone as “good” or “bad” for a specific atopic disorder is far too simplistic for the complexity of this topic. A recurring

theme in this rapidly growing literature—and that of ARI, wheezing, asthma, and COPD—is the heterogeneity of the study populations, the vitamin D regimens used, and the target outcomes. Until these issues are better addressed, it seems likely that we will continue to struggle to understand the exact link between vitamin D and atopy/allergy.

Nevertheless, there is sufficient evidence to suggest that sunlight and/or daily vitamin D supplementation has at least *some* role in preventing allergy, at least among those with vitamin D deficiency and other poorly defined host characteristics. Interestingly, each hypothesized benefit against the atopic/allergic disorders could be explained by vitamin D-mediated effects on innate immunity and the creation of a more salutary microbiome at the different epithelial surfaces—i.e., skin (AD), gut (food allergy), and airway (allergic rhinitis, asthma), with the airway microbiome perhaps altering risk of viral ARI [184]. The 2010 vitamin D-food allergy hypothesis article by Vassallo and Camargo [185] provides a useful example of the complexity that may underlie these different associations. Briefly, we proposed a “multiple hit” model for food allergy in which vitamin D deficiency in a developmentally critical period increases susceptibility to colonization with abnormal intestinal microbial flora and to gastrointestinal infections, contributing to abnormal intestinal barrier permeability and excess and inappropriate exposure of the immune system to dietary allergens. A compounding effect (and additional “hit”) of vitamin D deficiency is the promotion of a pro-sensitization immune imbalance that might compromise immunologic tolerance and contribute to food allergy. Similar models may apply to each of the vitamin D-atopy/allergy outcomes mentioned briefly here. The myriad effects of vitamin D—on lung development, innate immunity, and immunologic tolerance—also are relevant to our understanding of the relation of vitamin D to the different types of ARIs, asthma, and COPD.

## 8. Future research

Although vitamin D has effects on several important respiratory/allergy disorders, many scientific gaps remain. While all study designs can provide helpful information, the field has advanced greatly over the past decade and now needs to focus more on large, well-designed prospective cohort studies and RCTs; there is relatively little value from using a cross-sectional design to demonstrate (again) that people with very low 25(OH)D tend to have worse illness than others with “normal” levels of 25(OH)D. Moreover, future cohort studies and RCTs need to be large enough to explore likely interactions by participant characteristics, and,



for RCTs, by type of vitamin D intervention. While the mainstream media tends to present vitamin D in stark terms—such as amazing, dangerous, or useless—scientists need to avoid these unhelpful simplifications. The more we learn, the clearer it is that there are no easy answers [186]. To that end, I hope that trialists will see the value of the cited IPD meta-analyses on RCTs of vitamin D supplementation and ARI [162,163], asthma exacerbations [132], and AECOPD [175]. This type of international collaboration can help investigators to identify areas of greatest public health potential. Likewise, these collaborations can identify critical areas of uncertainty or topics for which additional studies are unlikely to be helpful.

In that spirit, I suggest below several questions that I think merit further investigation. The ideas are presented using the organizational structure of this chapter; the ordering is not meant to convey anything about their relative importance or feasibility.

## 8.1 Lung development

Are the animal model findings on vitamin D deficiency and lung development (and function) relevant to human lungs? If yes, what is the gestational and postnatal vitamin D status that provides optimal lung health, both in the short-term (with a focus on ARI, wheezing, and asthma) and in the long term (with a focus on incident COPD)?

## 8.2 Acute respiratory infection

- Why is there a strong inverse association between cord blood 25(OH)D and childhood wheezing in some birth cohorts but not in others? Are we missing important effect modifiers?
- Does vitamin D supplementation have different effects on the different microorganisms that underlie the composite outcome of ARI?
- Among individuals with vitamin D deficiency, is daily dosing better than nondaily dosing (including weekly) for preventing ARI?
- Why is bolus dosing ineffective for preventing ARI? Are the beneficial effects of vitamin D supplementation lost or are they offset by some negative feedback loop or new harm? Does a large initial bolus truly negate the observed benefits of daily dosing for preventing ARI?
- What is the target 25(OH)D level for a supplementation regimen that aims to prevent ARI? Is there value in targeting free 25(OH)D or circulating levels of cholecalciferol? (Although this chapter has not addressed these biomarkers, given very limited data on their role in respiratory/allergy disorders, the

detection and assay of vitamin D metabolites are discussed further in [Chapters 48–50](#).

## 8.3 Asthma

### 8.3.1 Asthma pathogenesis

- While most primary prevention studies have focused on prenatal vitamin D supplementation only, does vitamin D supplementation during both pregnancy and early childhood affect the risk of incident asthma?
- Is vitamin D status in adulthood associated with the risk of adult-onset asthma?
- What is the role of genetic factors in the hypothesized link between vitamin D and asthma?

### 8.3.2 Asthma control, including exacerbations

- What are useful biomarkers to identify “responders” to the likely beneficial effect of vitamin D supplementation on corticosteroid effectiveness in asthma?
- What is the effect of vitamin D supplementation on inhaled corticosteroid effectiveness and the prevention of asthma exacerbations in symptomatic children with asthma?
- Most of the questions listed under [Section 9.2](#) of this chapter also apply to asthma exacerbation.

## 8.4 COPD

### 8.4.1 COPD pathogenesis and progression

- Is vitamin D deficiency during pregnancy, early childhood, or adulthood associated with the risk of incident COPD?
- Given the required duration of these longitudinal studies, primary prevention research is likely to benefit from concurrent efforts based on animal models of COPD [187,188].
- What is the role of genetic factors in the hypothesized link between vitamin D and COPD?
- Is vitamin D deficiency associated with faster lung function decline among patients with COPD?

### 8.4.2 AECOPD

- What COPD subgroups, if any, demonstrate an inverse association between vitamin D status and risk of AECOPD?
- If such subgroups exist, what is the effect of daily vitamin D dosing (without initial bolus) on preventing AECOPD? What are the effects of vitamin D supplementation on the noninfectious aspects of AECOPD, such as concurrent heart failure?
- Most of the questions listed under COPD also apply to AECOPD.



## 8.5 Potential mechanisms

### 8.5.1 Immunity

The immunologic effects of vitamin D are a major focus of research and will continue to be so for many years. This research is discussed further in several chapters from [Section 11](#) of this volume. From the perspective of respiratory disorders, the role of vitamin D on innate immunity (e.g., hCAP-18) is of perhaps the greatest interest. If antimicrobial peptides do not mediate the observed reduction in vital ARI, how does vitamin D lower infectious disease risk?

Immunity researchers also are increasingly interested in an earlier and potentially larger question: how does sunlight itself affect immunity and do these effects mediate some of the health benefits that we now attribute to vitamin D? [189]. While basic research is critical for understanding this issue, so are RCTs. For example, when a well-designed and successfully implemented RCT of vitamin D supplementation fails to deliver the expected health benefit, there are many possible explanations, such as enrollment of a nonresponder population or use of a suboptimal vitamin D regimen. However, another explanation is that the value of 25(OH)D levels in the originally reported epidemiological association may have simply reflected its ability to quantify sunlight exposure, which actually was the driving force behind the observation; allocating people to vitamin D and placebo would not change the sunlight exposure that actually was responsible for the desired health effect.

Likewise, when RCTs of vitamin D supplementation are consistent with observational findings, these RCTs confirm, at last at some level, that vitamin D really does contribute to the observed effect. Of course, most associations between vitamin D status and health probably are a mix of phenomena, including both the direct effects of the sun and the direct effects of the vitamin D hormone. For each specific disease (or immunologic outcome), the causal mix could vary—e.g., 20% from sunlight itself versus 80% from vitamin D, or vice versa. The causal mix of sunlight and vitamin D might also vary from person to person, based on individual differences in environment, genes, and lifestyle. While this sunlight versus vitamin D issue may come to a head in terms of immunity research, the lessons learned are likely to apply to many diverse health outcomes.

### 8.5.2 Atopy and allergies

Observational studies report dose-dependent differences in how vitamin D status is associated with atopy/allergy disorders [87–90], which received recent support from a Finnish RCT [190]. Briefly, the authors reported that giving daily vitamin D (400 vs. 1200 IU) from age 2 weeks onward did not provide benefit; on the

contrary, infants randomized to the higher dose had a higher risk of milk allergy and there was increased risk of allergic sensitization among infants with high cord blood vitamin D status. Thus, at least for these atopic disorders, current data appear inconsistent with a simple linear association; simply put, “more is not always better.” For example, it seems likely that both low and high levels of vitamin D during infancy increase the risk of IgE sensitization. This curvilinear pattern would be consistent with how most hormones work, where optimal health requires that the hormone be neither too high, nor too low; instead, the hormone level needs to be somewhere “healthy” between. Although nonlinear associations may also have noncausal explanations, they are a growing focus of vitamin D research. Future studies are likely to demonstrate a relatively broad range of 25(OH)D values that lie in this risk nadir and to show that different diseases have different curvilinear associations. As our indoor lifestyle continues to drive vitamin D status downward, and public enthusiasm for vitamin D supplementation continues to drive 25(OH)D levels higher than are possible with sunlight alone [191], the curvilinear nature of these associations is likely to become increasingly apparent.

The complex association(s) between vitamin D and atopy/allergy are starting to advance an important discussion within the field. Unlike the “traditional” adverse effect monitoring of vitamin D trials in older adults—which focuses on hypercalciuria and hypercalcemia, two rare events until subject reaches extremely high 25(OH)D levels [191]—the observed increases in atopy/allergy risk may start at maternal or infant 25(OH)D levels as low as 40–50 ng/mL. Ongoing studies of vitamin D supplementation during pregnancy and infancy should help researchers to better understand this “new” potential risk, and this, in turn, may affect the use of vitamin D for the prevention and treatment of ARI and other respiratory disorders. The emerging atopy/allergy data encourage a more nuanced decision about vitamin D use and regimen, based not only on individual choices about risk, but on the individual’s environment, genes, and lifestyle. Indeed, recent studies confirm large interindividual variations in serum 25(OH)D response to vitamin D supplementation based on these individual factors [192]. This more nuanced decision-making will help to bring vitamin D supplementation away from the current “one size fits all” approach and into the realm of precision medicine [193].

## 9. Conclusion

Over the past 25 years, many studies have linked vitamin D status with common respiratory disorders, such as childhood wheezing, asthma, and COPD. While

vitamin D probably contributes to human lung development, there is little evidence that vitamin D deficiency affects the risk of developing childhood asthma and sparse data regarding the primary prevention of COPD. By contrast, vitamin D deficiency appears to increase the risk of ARIs, which cause most childhood wheezing and most exacerbations of asthma and COPD. Vitamin D's myriad effects on innate and adaptive immunity provide biological plausibility for how vitamin D deficiency: (1) increases the risk of infection, (2) decreases therapeutic response to corticosteroids; and (3) alters the risk of other related atopic conditions, such as atopic dermatitis, food allergy, and allergic rhinitis.

Further studies, especially well-designed RCTs [158], are needed to clarify the effects of vitamin D supplementation on ARIs and other respiratory disorders, particularly among individuals with vitamin D deficiency. For the primary prevention of asthma and COPD, prospective cohort studies continue to have value—e.g., to identify the characteristics of individuals who are most likely to benefit from a pregnancy or early life intervention with vitamin D. In the case of asthma, future RCTs should expand the vitamin D intervention beyond pregnancy and into early childhood, when children actually start to manifest asthma. The primary prevention of COPD is more complicated given its late onset; longitudinal studies will require decades to reach their primary outcome. Accordingly, primary prevention research may need to focus more on animal models of COPD.

For the prevention of ARI, asthma exacerbation, and AECOPD, I believe that future trials should use a daily supplementation regimen of vitamin D (without bolus) and have sufficient statistical power to determine if vitamin D can prevent (or reduce the severity of) these outcomes, both overall and in major subgroups. Recent IPD meta-analyses [162,163], support ARI prevention in at least subsets of the population. Likewise, the totality of the evidence supports the benefit of vitamin D supplementation in the management of asthma [132] and COPD [175]. However, the optimal person for a vitamin D intervention, and the optimal intervention itself, remain under study. Recent null RCTs remind us that there are no easy answers. At this time, however, I believe the totality of the scientific evidence suggests that correction of vitamin D deficiency is likely to lower the risk of ARI and improve the long-term management of asthma and COPD.

## 10. Summary points

- Vitamin D deficiency has been linked to childhood wheezing, asthma, and COPD.
- This does not appear to be due to effects on lung development but instead may be due to impaired immune responses to ARI.
- Improved supplementation trials are required to confirm a causative role of vitamin D in respiratory disease.

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## Vitamin D and acute illness

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### OBJECTIVES

- Understand the association between low vitamin D and increased adverse outcomes in the critically ill.
- Identify factors that contribute to low vitamin D status in the critically ill population.
- Appreciate the importance of heterogeneity of critical illness in the interpretation of vitamin D supplementation trials.

### 1. Introduction

Low vitamin D levels are estimated to be present in 30%–50% of the general population [1] and is associated with increased mortality risk [2]. Vitamin D deficiency is observed worldwide and in countries where food fortification of vitamin D and supplemental intake is common. Factors associated with higher risk of low 25-hydroxyvitamin D (25(OH)D) levels include older age, living in the far Northern and Southern hemispheres, low exposure to UVB radiation via sunlight, season, altitude, darker skin pigmentation, and low dietary or supplemental vitamin D intake [3]. Data show that low vitamin D levels are common in the critically ill and are associated with increased mortality and adverse events [4–11].

Patients who are admitted to intensive care units (ICUs) have high mortality. Over the past 30 years, our

care delivery has improved so that previously fatal illness presentations are increasingly survivable. Those ICU patients who survive hospitalization have high risk for mortality and lower quality of life for up to a year after hospital discharge [12,13]. ICU stays are complicated by muscle weakness, critical illness polyneuropathy, and alterations in organ function, which contribute to the reductions in quality of life witnessed in ICU survivors [14]. Powerful data exist showing that ICU survivors have lower health-related quality of life at baseline and at 6 months to 1 year after hospital discharge [13]. As vitamin D receptors (VDRs) and vitamin D metabolic enzymes are distributed widely, vitamin D is an attractive potential modifiable target for critical care outcomes improvement. Indeed, data are emerging relating vitamin D supplementation and critical illness outcomes. The VITdAL-ICU trial [15], a single-center, randomized, double-blind, placebo-controlled trial of 475 critically ill medical and surgical subjects, though negative overall, did show in a secondary outcome that high-dose oral vitamin D<sub>3</sub> significantly improved mortality in patients with severe vitamin D deficiency. The VIOLET trial [16], a multicenter, randomized, double-blind, placebo-controlled phase III trial of 1360 subjects at high risk for ARDS and mortality showed no benefit with high-dose enteral vitamin D<sub>3</sub> supplementation.

This chapter aims to present the current knowledge on vitamin D status in critically ill patients. Vitamin D issues relevant to the critically ill will be highlighted from risk factors to the epigenetics of vitamin D and data from interventional trials.

## 2. Prevalence of vitamin D deficiency in the critically ill patient

With the high level of interest in vitamin D as a potentially modifiable risk factor for critical illness outcome, a number of studies now exist illustrating the epidemiology of serum 25(OH)D levels in the ICU. The potential role of vitamin D in the critically ill has become clearer with a large number of clinical research investigations worldwide. Variability exists in published studies regarding the prevalence of vitamin D deficiency or inadequacy [17]. No universal definition exists for optimal vitamin D status, and existing data highlight comparability problems between 25(OH)D assays [18]. As the serum half-life of 25(OH)D is approximately 3 weeks, total vitamin D status is determined by measurement of 25(OH)D rather than other vitamin D metabolites. Furthermore, 25(OH)D correlates positively with calcium absorption efficiency, unlike circulating levels of active, hormonal 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) [19].

Consensus statements and experts differ regarding the optimal 25(OH)D level threshold with the Institute of Medicine at 20 ng/mL [20] and the Endocrine Society at 30 ng/mL [21], and most studies will utilize these cut-points. Vitamin D sufficiency can be thought of as a circulating 25(OH)D level that is adequate for physiologic needs. A sufficient 25(OH)D level should maximally suppress circulating parathyroid hormone (PTH), promote calcium absorption, and is associated with the highest bone mineral density. Low 25(OH)D is present in many patient populations worldwide: in young, middle-aged, and elderly adults, particularly in those of African descent [22]. In the United States, most patients have 25(OH)D levels <30 ng/mL [23].

Importantly, 25(OH)D levels deemed sufficient in the population may not be adequate in the critically ill. ICU-based vitamin D studies are not standardized in terms of deficiency cut-points, time of 25(OH)D measurement, or 25(OH)D assay utilized. Abnormal 25(OH)D levels are often variably defined as insufficient, deficient, and severely deficient. A high proportion of critically ill medical and surgical patients have low 25(OH)D levels. Observational data indicate that it is common to find over 50% of ICU cohort patients have 25(OH)D levels that are lower than 30 ng/mL. Studies worldwide report a high prevalence of abnormally low 25(OH)D levels in critically ill patients with different case mixes [4,5,7,9,10]. The prevalence of low 25(OH)D levels in critically ill patients is substantially higher than what is reported in general medical wards [24].

## 3. Vitamin D deficiency risk factors in the critically ill

### 3.1 Ethnicity

Studies in the general population as well as the critically ill demonstrate that dark skin pigmentation is a risk factor for vitamin D deficiency [6]. The synthesis of vitamin D depends on the skin melanin concentration. Individuals with high skin melanin concentration have a less efficient conversion of 7-dehydrocholesterol to previtamin D<sub>3</sub> as melanin absorbs and scatters UV-B. Thus, individuals with dark skin pigmentation have slower vitamin D synthesis than light skin pigmentation (see Chapters 2 and 3). Comparing patients with different skin pigmentation, an association is found between light skin pigmentation and higher 25(OH)D levels. In the critical care environment, patients with dark skin pigmentation have higher proportion of low vitamin D levels relative to those with light skin pigmentation [6]. Vitamin D-binding protein (DBP) allelic combinations are demonstrated to occur at different frequencies in different ethnic groups (see Chapter 7). Despite these differences, there is no difference in the effect of oral vitamin D on serum 25(OH)D levels relative to ethnicity [25–27], an important issue in vitamin D supplementation trials in the ICU.

### 3.2 Seasonality and sun exposure

Most vitamin D synthesis occurs via the skin following UV-B radiation exposure. A single minimal erythema dose exposure (10–12 min of peak summer sun exposure in a light skin pigmentation individual with majority of skin exposed) releases 10,000–20,000 IU vitamin D into the circulation within 24 h [28]. Seasonal variations can be demonstrated in 25(OH)D levels in patients [5,9] (see Chapter 56). Critically ill patients admitted in spring reflect the low sunlight exposure of winter and have lower levels of 25(OH)D and are more often severely deficient (25(OH)D < 15 ng/mL) than in the summer and fall [6,9]. Living at low latitude does not preclude one from low 25(OH)D levels but provides different sunshine periods, more UV-B exposure, and is a potential study design issue regarding the geographic heterogeneity of 25(OH)D levels [29]. Patients at higher altitudes have a more intense UV-B exposure due to the shortened path length of UV-B needs to travel to reach a patient's skin. This results in higher vitamin D production [3].

Sunscreen usage prevents UV-B and partially prevents UV-A radiation from hitting the skin. In studies, sunscreen usage appears to reduce vitamin D synthesis [30] by more than 95% with a sun protection factor (SPF) of 8 and over 98% with an SPF of 15 [31]. Likely from sporadic usage patterns and the general good health of a sunscreen usage population, no study has shown that sunscreens cause vitamin D deficiency. Sunscreen use does not appear to be associated with vitamin D deficiency in the general population [32].

### 3.3 Chronic disease

Chronic disease is associated with alterations in vitamin D status. Conditions that alter intestinal absorption, fat storage, or metabolism by the enzyme 25-hydroxylase can alter vitamin D bioavailability [33]. Kidney production of 1,25(OH)<sub>2</sub>D by the enzyme 25(OH)D-1 $\alpha$ -hydroxylase (1 $\alpha$ -hydroxylase, CYP27B1) is decreased proportionally to the severity of chronic kidney disease mirrored by decreases in circulating 1,25(OH)<sub>2</sub>D [34] (see Chapter 16 and Chapter 79). Patients with higher renal function as measured by creatinine clearance have higher serum 1,25(OH)<sub>2</sub>D levels [35]. With end-stage renal disease 1,25(OH)<sub>2</sub>D may be undetectable depending on residual kidney function. The proteinuria of nephrotic syndrome results in DBP-bound 25(OH)D losses in the urine [36].

Vitamin D<sub>3</sub> is 25-hydroxylated in the liver to produce 25(OH)D. VDR gene polymorphisms may predispose patients to autoimmune liver diseases including primary biliary cirrhosis and autoimmune hepatitis and may be associated with disease severity [37]. Vitamin D deficiency is common with cholestatic liver diseases promoting the European Association for the Study of the Liver to publish guidelines for vitamin D supplementation in this population. Reduction of bile salt availability decreases absorption of the lipophilic vitamin D [38]. Low vitamin D levels are almost universal in patients with chronic liver disease, with a third of patients having 25(OH)D < 12 ng/mL with a higher prevalence in cirrhosis [39] (see Chapter 77).

In published studies, body mass index (BMI)  $\geq$  30 is consistently associated with low 25(OH)D levels. It is postulated that “sequestration” of vitamin D by adipose tissue contributes to the observed low 25(OH)D in patients with BMI  $\geq$  30 [40]. Adipose tissue has the vitamin D receptor and the ability to synthesize 1,25(OH)<sub>2</sub>D. Low 25(OH)D may relate to obesity by the alteration of lipogenesis and/or the regulation of adipose tissue differentiation, mass, and metabolism [41]. BMI is a relatively crude index of adiposity, as it does not quantify

body composition [42]. When serum 25(OH)D values are adjusted for body weight, the difference between patients with BMI  $\geq$  30 and BMI 25–30 is no longer present [43]. Furthermore, it appears that low 25(OH)D may be associated more with markers of glucose homeostasis (insulin resistance, blood glucose) rather than with high BMI [44].

### 3.4 Medication usage

Interactions between medications and vitamin D metabolism and action exist. The pregnane X receptor is a master transcription factor for cytochrome P450 3A4 (CYP3A4), an enzyme important for xenobiotic and drug detoxification. Activation of the pregnane X receptor upregulates 24-hydroxylase (CYP24A1), which increases catabolic degradation of 25(OH)D and 1,25(OH)<sub>2</sub>D, leading to low 25(OH)D levels [45]. Activators of the pregnane X receptor include antiepileptics, antiinflammatory agents, antiretroviral drugs, and the herbal medicines, specifically phenytoin, carbamazepine, cyclophosphamide, taxol, tamoxifen, clotrimazole, rifampicin, dexamethasone, nifedipine, spironolactone, ritonavir, saquinavir, cyproterone acetate, kava, and St. John’s wort [45].

### 3.5 Severity of critical illness and organ failure

ICU patients commonly present with acute-on-chronic organ failure or severe comorbidities, which could relate to the high prevalence of low 25(OH)D observed at ICU admission [6]. This is especially relevant for patients with chronic kidney disease and chronic liver disease as previously noted. Observational studies show a strong link between low 25(OH)D and acute disease severity. Investigators have found that low 25(OH)D is associated with Simplified Acute Physiology Score II (SAPS II) [4], the Sequential Organ Failure Assessment (SOFA) score [4,9,46], Acute Physiology and Chronic Health Evaluation (APACHE) II score [46], and the number of acute organ failures [6]. These findings are not surprising as the level of DBP drops in acute inflammatory conditions common in the critically ill and likely reflects the severity of illness. Partial release of 25(OH)D from the DBP is a likely source of variability for immunoassays commonly used to determine 25(OH)D. Patients with inflammation and sepsis have lower DBP concentrations, which may factor in the low 25(OH)D levels observed in ICU patients when measured with LC-MS/MS [47].



#### 4. Vitamin D mechanisms in critical illness

Vitamin D status affects a large number of biological processes vital to the critical illness response, including gene expression, protein synthesis, metabolism, signal transduction, and cellular proliferation. The mechanism of action of vitamin D involves  $1,25(\text{OH})_2\text{D}$ , VDR and its associated transcriptional coactivators, plasma transport by DBP, the vitamin D-activating cytochrome P450s (CYP27A1, CYP27B1 and CYP2R1), and the vitamin D-catabolic cytochrome P450 CYP24A1 (see Chapters 4–13). A photosynthetic mechanism in human skin via UV-B radiation converts 7-dehydrocholesterol to previtamin  $\text{D}_3$ , and then to vitamin  $\text{D}_3$  via thermally induced isomerization. Vitamin  $\text{D}_3$  is subsequently hydroxylated to  $25(\text{OH})\text{D}$  in the liver and again in the kidney to form biologically active  $1,25(\text{OH})_2\text{D}$ . When vitamin D enters the circulation after UV-B generation, it is coupled primarily with DBP. In contrast, after intestinal absorption, vitamin D enters the circulation and is coupled with both DBP and lipoproteins [48]. Additionally, autocrine production of  $25(\text{OH})\text{D}$  exists in tissues containing the vitamin D 25-hydroxylase [49]. Activation of human monocytes or macrophages by ligands of Toll-like receptors, IFN- $\gamma$  or CD40 ligand, results in upregulation of  $1\alpha$ -hydroxylase and nuclear VDR expression and increased conversion of  $25(\text{OH})\text{D}$  to  $1,25(\text{OH})_2\text{D}$  [50]. Vitamin D, produced in the skin or from intestinal uptake, is delivered primarily to the liver, hydroxylated to  $25(\text{OH})\text{D}$ , which is then associated with DBP and then released to the circulation [51].

The main serum carrier of  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  is DBP, while albumin can act as a low-affinity binder [52]. The pharmacokinetics and distribution of vitamin D metabolites are determined by lipophilicity and the relative activities of vitamin D synthetic and catabolic enzymes [53].  $1,25(\text{OH})_2\text{D}$  is a powerful inducer of CYP24A1 expression and 24-hydroxylase activity and  $24,25(\text{OH})_2\text{D}$  formation, a metabolite of  $25(\text{OH})\text{D}$  of unclear function [54], although 24-hydroxylase can also catabolize  $1,25(\text{OH})_2\text{D}_3$  [55].

Inflammation is associated with decreased levels of DBP [56,57]. Additionally, DBP levels fall with severe tissue and cell damage. The extent of DBP reduction is associated with the acuity of illness with low DBP levels noted in critically ill patients with acute organ dysfunction, respiratory failure, hematologic failure, sepsis, and ARDS [58]. Importantly for the critically ill, DBP also has activity in actin scavenging, macrophage activation, and fatty acid transport [59]. With apoptosis, cell death and tissue injury actin are released systemically into the circulation. Outside of the cell, G-actin polymerizes into F-actin filaments, which may contribute to multiple

organ dysfunction syndrome. DBP binds to G-actin monomers accentuating G-actin clearance via the liver [60].

The phosphaturic hormone FGF23 is produced by osteocytes that plays an important role in vitamin D homeostasis [61] (see Chapter 19). FGF23 is a  $1\alpha$ -hydroxylase inhibitor and a 24-hydroxylase stimulator resulting in decreased activation of  $25(\text{OH})\text{D}$  and increased  $25(\text{OH})\text{D}$  and  $1,25(\text{OH})_2\text{D}$  catabolism [62]. FGF23 induces expression of CYP24A1 [62] at the same time that elevated PTH inhibits kidney expression of CYP24A1 [63]. In critically ill patients, the interplay between  $25(\text{OH})\text{D}$ ,  $1,25(\text{OH})_2\text{D}$ ,  $24,25(\text{OH})_2\text{D}$ , FGF23, and PTH is not well described.

VDR [64,65] and vitamin D metabolic enzymes [66,67] are present in tissues other than bone and the intestine, indicating that vitamin D is involved in the metabolism and function of many cell types. In particular, VDRs are expressed in T cells, activated B cells, neutrophils, and dendritic cells [68,69] (see Chapters 94–96). Vitamin D deficiency experimental studies have demonstrated defects in macrophage chemotaxis, phagocytosis, and the production of proinflammatory cytokines [70]. Vitamin D also links Toll-like receptor (TLR) activation and innate immunity [71] (see Chapter 94). Human macrophage stimulation by TLR induces  $25(\text{OH})\text{D}$  conversion to  $1,25(\text{OH})_2\text{D}$  as well as VDR expression and antimicrobial peptide production (see Chapter 9). Furthermore,  $1,25(\text{OH})_2\text{D}$  is important in upregulating genes essential for tight junctions, gap junctions, and adherens junctions, which promote barrier integrity [72].

The transcription of multiple inflammatory cytokines is influenced by  $1,25(\text{OH})_2\text{D}$  [73]. Human monocytes incubated with  $1,25(\text{OH})_2\text{D}$  have downregulated pathogen receptor molecules and inflammatory responses following exposure with *Candida albicans*, bacterial ligands, lipopolysaccharide (LPS), and lipoteichoic acid [74]. During human macrophage differentiation, differential expression of VDR and  $1\alpha$ -hydroxylase exist suggesting that vitamin D may encourage the differentiation of precursor monocytes to mature phagocytic macrophages [75].  $1,25(\text{OH})_2\text{D}_3$  is related to the oxidative burst potential via activation of human monocyte secretion of hydrogen peroxide [76] (see Chapter 9).

Vitamin D may modulate the immune response to sepsis via regulation through CYP24A1 and TLR. Activity of 24-hydroxylase, the catabolic enzyme of vitamin D, which attenuates responses to  $1,25(\text{OH})_2\text{D}$ , is functionally altered by splicing variants of CYP24A1 [77].  $1,25(\text{OH})_2\text{D}$  also downregulates monocyte TLR2 and TLR4 expression, which may blunt the inflammatory response to TLR ligands providing a potential negative feedback regulation [78].

Data indicate that vitamin D also influences adaptive immunity through differentiation of inflammatory T helper 17 (Th17) T cell subsets and regulatory T cells (Treg cells) [79]. Via production of IL-17, Th17 cells accentuate inflammation and chemotaxis of neutrophils. Data show that Th17 cells can transdifferentiate into Treg cells, which are important for inflammation resolution and the beginnings of organ repair in critical illness [80]. Additional evidence lends credence to the hypothesis that Treg cells are central to inflammation resolution in animal models of acute lung injury [81]. Importantly, 1,25(OH)<sub>2</sub>D regulates expression of FOXP3 in CD4<sup>+</sup> T cells important for the development and functioning of Treg cells [82] (see Chapter 96).

Vitamin D exerts stimulatory effects on innate immunity and can also suppress hyperreactive immune response [71]. Biologic evidence shows that vitamin D promotes Treg cell induction, T helper 1 and 2 (Th1 and Th2) response modulation, Th17 cell suppression, and the regulation of dendritic cell maturation [79]. In a rodent model of sepsis, pretreatment with vitamin D showed lower decrements in platelet counts, while another shows improved short-term survival rates [83,84]. In other experimental models of sepsis, 1,25(OH)<sub>2</sub>D administration was associated with improved blood coagulation parameters in sepsis-associated disseminated intravascular coagulation [85]. Experimental meningoencephalitis caused by *Escherichia coli* was associated with worse outcomes in animals with low vitamin D [86]. In experimental peritonitis, vitamin D deficiency significantly increased systemic, local, and bronchoalveolar lavage quantitative bacterial culture [87]. VDR null mice have heightened mortality following LPS-induced endotoxemia [88]. Human endothelial cells exposed to 1,25(OH)<sub>2</sub>D have an attenuated inflammatory response following LPS administration [89].

## 4.1 Antimicrobial peptides

An important role of vitamin D is the upregulation of antimicrobial peptide (AMP) production in response to various pathogens via the innate immune system [90]. AMP production occurs within minutes of pathogen recognition. Human AMPs include cathelicidin, 6  $\alpha$ -defensins, and 4  $\beta$ -defensins. Specifically, vitamin D upregulates cathelicidin and  $\beta$ -defensins with broad-spectrum antimicrobial effects expressed in plasma as well as barrier tissues such as airway, bladder, and gastrointestinal epithelium [91] (see Chapter 94).

### 4.1.1 $\beta$ -2

The expression of  $\beta$ -defensin 2 occurs in airway epithelial cells and keratinocytes with induction

following inflammatory signals. 1,25(OH)<sub>2</sub>D induces genes that encode  $\beta$ -defensin 2 [92].  $\beta$ -2 activates innate immunity via activity in phagosomes or phagolysosomes [93].  $\beta$ -2 increases host resistance to gram-negative bacteria and *Candida albicans* [94]. In addition,  $\beta$ -defensin 2 increases host resistance to respiratory syncytial virus, influenza A virus, para influenza virus, and adenovirus [95]. Further,  $\beta$ -defensin 2 is increased in the GI epithelium as a barrier response to potential pathogens [96].

### 4.1.2 Cathelicidin

Cathelicidin is produced by cells that are in contact with the environment (mucosal epithelium, keratinocytes) and cells of the innate immune system [97]. VDR activation is important for cathelicidin production as a vitamin D response element (VDRE) sequence is present in the promoter region of the cathelicidin gene [98]. Cathelicidin regulates inflammatory responses, accentuates chemotaxis, promotes phagocytosis, increases vascular permeability, improves wound healing, and promotes the production of reactive oxygen species, the disruption of bacterial biofilm, and the neutralization of lipopolysaccharides [91,99]. Cathelicidin also is noted to increase cell stiffness and decrease cell permeability in the lung epithelium resulting in decreased cell infection [100]. Cathelicidin can recruit and induce proliferation of endothelial progenitor cells to wound sites during healing. Importantly, cathelicidin regulates apoptosis of epithelial cells and neutrophils extending the time of chemokines and cytokines production and microbe clearance [101]. Further, it is demonstrated that cathelicidin binds to self-DNA a potent activator of innate immunity [102]. Cathelicidin is rapidly upregulated in urinary epithelial cells in response to infection [103] and in skin epithelial cells quickly following wounding [104]. The activated form of cathelicidin, LL-37, has direct microbicidal effects on vancomycin-resistant enterococci, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* [105]. LL-37 kills bacteria by membrane permeation and disruption by the helical region [106]. In vitro, LL-37 binds *Escherichia coli* LPS, which suggests that LL-37 may serve to neutralize low levels of circulating LPS [105].

Experimentally, the effect on pathogens by vitamin D varies. In vivo, CD14<sup>+</sup> cells increase 1 $\alpha$ -hydroxylase expression following infection [107]. In a study of postmenopausal women supplemented with 2000 units of daily oral 25(OH)D<sub>3</sub> for 12 weeks, bladder biopsy tissue obtained at week 0 and week 12 showed a significant increase in cathelicidin expression over time with experimental infection with uropathogenic *E. coli* [108]. In a study on human airway epithelial cells, cathelicidin

production was induced by vitamin D with enhanced capacity to kill *Pseudomonas aeruginosa* and *Bordetella bronchiseptica* [109]. The antimicrobial effect is not universal. In vivo experiments show that LL-37 is not induced in human monocytes following salmonella bacterial infection and clearance [110]. In experimental colitis, 1,25(OH)<sub>2</sub>D pretreatment increased susceptibility to *Citrobacter* via mucosal Th17 responses suppression [111]. Further, experimental group A streptococcal exposure to low-dose LL-37 caused resistance to killing by human neutrophils [112].

While the evidence for the role of cathelicidin in innate immunity is strong, it is not certain if plasma LL-37 is a marker of the effect of vitamin D. In study volunteers, a positive correlation exists between serum 25(OH)D and free LL-37 levels when serum 25(OH)D concentrations < 32 ng/mL [113,114]. A vitamin D supplementation study in healthy volunteers showed the greatest increase in free LL-37 levels occurred in subjects with the highest increase in 25(OH)D [113]. In the ICU patient population, a significant positive correlation exists between 25(OH)D serum levels and cathelicidin production [56]. In a study of 112 hospitalized patients with community-acquired pneumonia, cathelicidin levels were not associated with 25(OH)D [115]. In the dialysis population, associations exist between cathelicidin and infectious disease outcome [116]. In the ICU patient population, a significant positive correlation exists between 25(OH)D serum levels and cathelicidin production as well as associations between low cathelicidin and increased mortality [56,117].

#### 4.1.3 Inflammasome activation

In critically ill patients, pathogen-associated molecular patterns or host cell–derived damage-associated molecular patterns such as circulating mitochondrial DNA [118] and fatty acid synthase–dependent lipid synthesis trigger Toll-like receptors to initiate transcriptional priming of the NLRP3 inflammasome [119]. The NLRP3 inflammasome is a multiprotein complex that promotes caspase-1 activation and cleavage of pro-IL-1 $\beta$  and pro-IL-18 to intensify inflammation [120]. Vitamin D may be important for NALP3 inflammasome regulation [121] and autophagy [92]. Human-derived peripheral blood mononuclear cell (PBMC) studies related to vitamin D regulation of TLR-2, TLR-4, GATA-3, VDR, pp38, ERK1/2, and p65 are supportive of an antiinflammatory effect of vitamin D [74,122]. By extension, it is possible that vitamin D supplementation in critical illness blunts inflammasome activation via preservation of mitochondrial integrity based on structural studies that show mitochondrial alterations and lower mitochondrial number in sepsis nonsurvival [123].

Bacterial toxins, pathogen-associated molecular patterns, and reactive oxygen species (ROS) trigger the association of the NALP3 inflammasome with thioredoxin-interacting protein (TXNIP) in human macrophages, which may be regulated by vitamin D [121]. Recent in vitro experiments suggest that vitamin D<sub>2</sub> analog treatment may depress TXNIP mRNA and protein expressions [124]. Additionally, thioredoxin (TRX) is important for redox regulation, interacts with TXNIP, and is downregulated by vitamin D<sub>3</sub> [125]. It is further observed that vitamin D can upregulate NOD2 [92], an NLR protein in the same family as the inflammasome NALP3. Studies have linked NOD2 function to autophagy [126,127], and autophagy proteins regulate innate immunity via inhibition of mitochondrial DNA (mtDNA) release via by the NALP3 inflammasome [128]. Mitochondrial dysfunction and an impaired autophagic response are reported to be associated with worsened mortality from murine sepsis [128]. Recently, in two critical ill cohorts, it was shown that elevated circulating mtDNA levels are a robust predictor of increased 28-day mortality in medical ICU patients [129].

Autophagy is a lysosome-mediated catabolic pathway and core cellular housekeeping processes to ensure synthesis, degradation and recycling of macromolecules, and inclusions in mononuclear cells [130]. Autophagy is an inducible response to stress triggered by hypoxia, hyperoxia, pharmaceuticals, oxidative stress, proinflammatory states, and endoplasmic reticulum stress [131]. Autophagy also regulates innate immune responses [128]. Vitamin D signaling appears to regulate autophagy at different steps, including induction, nucleation, elongation to maturation, and degradation. Cathelicidin induces autophagy activation and autophagolysosome formation in human monocytes/macrophages [130]. Additionally, vitamin D signaling regulates autophagy to decrease autophagy-related damage and to decrease invasion of microorganisms [132].

#### 4.2 Metabolomics, transcriptomics, and epigenetics of vitamin D

In critical illness, severe disruption of many metabolic pathways occurs with loss of metabolic homeostasis [133]. Analyzing metabolites at a particular time point gives a picture of the functional product of the relationship between genes, transcription, proteins, and environmental factors [134]. Critical illness severely disrupts homeostasis affecting a large number of metabolic pathways [133]. In the critically ill, metabolic profiles are the end product of the response at the cellular level to injury, inflammation, infection, hypoxia, oxidative stress, and micronutrient depletion [135]. However,



the metabolic consequence of low vitamin D status in the critically ill is not known. Vitamin D has extensive influence on apoptosis, nuclear transcription as well as cell cycle regulation and differentiation [136]. The tissue distribution of vitamin D receptors and vitamin D metabolic enzymes is broad and underscores the importance of vitamin D in the function and metabolism of numerous cell types [137]. Vitamin D deficiency alters biological processes that are important for the immune response such as gene expression, cytokine production, cellular function, and metabolism.

Evidence supports different metabolomic profiles in ambulatory patients who respond to vitamin D supplementation compared with those who respond poorly [138,139]. Studies are emerging that consider the influence of vitamin D on the critical illness metabolome [140–142]. Significant differences exist in the metabolome relative to vitamin D status early in critical illness [140]. With high-dose vitamin D<sub>3</sub> intervention, patterns of metabolite change during early critical illness differ according to the 25(OH)D level response [141]. Furthermore, women have significantly different pharmacokinetics and metabolic responses to high-dose vitamin D<sub>3</sub> intervention compared with men [142].

Gene expression during critical illness is regulated by epigenetic mechanisms, which modify inflammatory responses and host defense (see Chapter 12 and Chapter 14). The epigenetic effect of vitamin D is primarily covalent modification of histones by acetylation [143]. Vitamin D mediates an epigenetic effect via binding with the VDR. Vitamin D both regulates epigenetic events and also is itself regulated by epigenetic means. The vitamin D signaling system genes coding for the enzymes 25-hydroxylase, 1 $\alpha$ -hydroxylase, 24-hydroxylase, and the VDR can be silenced by DNA methylation due to the CpG islands located in the promoter regions [144]. VDR protein physically interacts with coactivator and corepressor proteins. This is then followed by interaction with chromatin modifiers (histone acyltransferases, histone deacetylases, histone methyltransferases) as well as with chromatin remodelers (histone demethylases of the Jumonji C-domain containing proteins, and lysine-specific demethylase families) [145].

Low 25(OH)D in some patients has been found to be related to specific genetic variants [146]. Single-nucleotide polymorphisms (SNPs) in vitamin D activation or degradation enzymes as well as in the DBP can alter serum 25(OH)D concentrations and metabolites [146,147] (see Chapter 60). The vitamin D<sub>3</sub> supplementation response as measured by 25(OH)D levels is dependent upon baseline vitamin D status and high body mass index [148]. A genetic basis for vitamin D supplementation response may contribute to the large variability noted in 25(OH)D levels following equal vitamin D supplementation doses [149].

Genetic factors have a large influence on critical illness, specifically regulation of genes involved in immune response regulation. Susceptibility to and outcome of critical illness may be related to polymorphisms of genes involved in the expression of inflammatory cytokines, innate immunity pathways, and the coagulation cascade. The following polymorphisms may be significant for sepsis vulnerability and critical care outcomes: bacterial permeability increasing protein, CD14, IgG receptors, IL-10, IL-6, lipopolysaccharide binding protein, lymphotoxin alpha, macrophage migration inhibitory factor, mannose-binding lectin, plasminogen activator inhibitor-1, TLRs, and tumor necrosis factor may be important in sepsis susceptibility and outcomes [150].

Vitamin D regulates over 1000 genes, and genetic differences may contribute (albeit as a small percentage) to the wide variation found in serum 25(OH)D levels (see Chapter 60). DBP and VDR SNPs are noted to correspond to alterations in vitamin D metabolism and altered immune response to infections [151,152]. SNPs at restriction enzyme sites D432E and T436K are known to change the VDBP protein structure. This altered structure is found in the common Gc1f, Gc1s, and Gc2 variants of VDBP, which alter the VDBP to 25(OH)D-binding affinity and bioavailability and subsequently modify LL-37 induction [151,152]. In the VDR, SNPs at restriction endonuclease sites for Taq1, Bsm1, and Fok1 restriction enzyme sites are associated with infectious disease [153]. The Fok1 SNP in the VDR gene affects VDR signaling in immune cells resulting in dampening of the cytokine response to inflammation [154].

## 5. Vitamin D and critical care patients

### 5.1 Assays for vitamin D

Circulating 25(OH)D is difficult to measure as it is highly hydrophobic, strongly bound to DBP (dissociation constant,  $K_d$ ,  $5 \times 10^{-8}$  M); there is negligible non-protein-bound 25(OH)D in blood, and few of the available DBP-binding sites are bound to 25(OH)D. Comparing clinical studies that measure 25(OH)D in the ICU has limitations due to the differences in the assays to measure 25(OH)D. 25(OH)D assays utilize (1) extraction with organic solvents, reconstitution in a matrix, and then quantification via immunoassay or (2) separation via chromatography. The organic solvents that are necessary to separate DBP from 25(OH)D are not compatible with most protein-binding assays or immunoassays. The measurement of 25(OH)D is indirectly measured by competitive binding methods via protein-binding assays and immunoassays and directly



measured by chromatographic separation by liquid chromatography–tandem mass spectroscopy (LC-MS/MS) and high-performance liquid chromatography (HPLC) (see Chapter 48). Method comparison studies of various 25(OH)D assays show variability between methods [18,47] (see Chapter 49), and DBP concentration-dependent inaccuracies occur with 25(OH)D immunoassays [47]. Critically ill septic patients and those with inflammation or organ dysfunction have low DBP concentrations. Biologically active 25(OH)D is dependent on the 25(OH)D concentration and the protein isoform of DBP. These issues may be responsible for the low 25(OH)D concentration observed when measured with LC-MS/MS in critically ill patients [47,56].

Variation occurs in analysis of 25(OH)D levels due to reagent standardization, operator expertise but most importantly assay type. Standards for 25(OH)D assay type employed or cut-points for specific 25(OH)D assay used do not exist. Work performed toward standardization of 25(OH)D assays include the Vitamin D External Quality Assessment Scheme, US National Institute for Standards and Technology, and the University of Ghent. Due to assay variation, cut-points for determining “deficiency” will need to be assay specific. Due to assay variation and nonstandardization, it is important to take into consideration assay type when comparing clinical studies, interpretation of metaanalyses, and policy discussions regarding cutoffs for deficiency and supplementation of 25(OH)D. Please refer to Chapter 49 for a more detailed discussion of this topic.

## 5.2 Observational data

Mortality risk in the general population drops with increasing 25(OH)D levels, with the most favorable levels at 30–35 ng/mL. Vitamin D supplementation is associated with a small decrease in all-cause mortality [155]. High levels of 25(OH)D (>40 ng/mL) may be required for peak immune function [156]. A large meta-analysis on adult outpatients demonstrated that low 25(OH)D was associated with increases in all cause-mortality [157]. Mendelian randomization studies indicate that vitamin D deficiency increases mortality but only at very low levels of 25(OH)D [158,159]. In hospitalized patients, there is a relationship between vitamin D levels and important outcomes. Serum 25(OH)D concentrations <20 ng/mL in hospitalized adults are associated with unfavorable outcomes including heightened mortality [160]. Genetic differences in the vitamin D receptor may account for the vitamin D–clinical outcomes relationship [161].

### 5.2.1 Vitamin D and critical illness outcomes

A low vitamin D level in the critically ill (serum 25(OH)D ( $\leq 20$  ng/mL) is common being present in up to 50% of patients [4–11]. Low vitamin D levels in the critically ill are associated with increased infection severity and increase cost [5,162]. Low serum 25(OH)D appears to be a biomarker of severity of illness and critical illness outcomes and raises the possibility of vitamin D as a modifiable risk factor through supplementation [4–11]. It is postulated that low vitamin D levels may play a role in adverse critical illness outcomes, including metabolic derangement, infection, development, and outcome of sepsis and multiorgan failure [17,163,164]. Studies show an inverse relationship between vitamin D and severity of illness [4,9]. Critically ill patients with low vitamin D levels have an increase in all-cause mortality [4–11]. In ICU survivors, low vitamin D levels is related to higher likelihood of facility placement following hospital discharge [165].

### 5.2.2 Sepsis

Sepsis is a common cause of death worldwide and the most common cause of death among critically ill patients outside of coronary ICUs [166]. The yearly cost of care for hospitalized patients with sepsis is \$20 billion in the United States [167]. Observational data suggest that low vitamin D levels may contribute to sepsis development and sepsis outcomes primarily due to alteration in the innate immune responses [163].

Data from critically ill patients indicate that patients with low vitamin D (25(OH)D  $\leq 15$  ng/mL) have a higher risk for mortality, bloodstream infection, and sepsis [4–8,162,163]. In addition, low vitamin D levels in the critically ill are a strong predictor for organ dysfunction [5–7,163]. Low vitamin D prior to hospital admission is associated with sepsis susceptibility and increased mortality in septic patients [163]. Metaanalyses of 14 observational studies in the critically ill demonstrated that low vitamin D increases susceptibility for severe infection susceptibility and mortality [168].

### 5.2.3 Acute Kidney Injury

Critically ill patients with preexisting low vitamin D levels (25(OH)D  $\leq 15$  ng/mL) have higher risk of development of acute kidney injury (AKI) [7]. Experimental models of renal ischemia reperfusion (IRI) show that low vitamin D levels accentuate AKI [169]. In particular, rats with low vitamin D have a more pronounced drop in glomerular filtration rate, higher urinary protein excretion, attenuated renal aquaporin 2 expression, and more substantial histological damage (specifically tubular necrosis) following IRI [169]. Experimental IRI in the context of low vitamin D shows increased

inflammation and fibrosis, lower renal repair responses, and more pronounced changes in renal capillary density [170].

Vitamin D supplementation prior to experimental LPS-induced AKI results in attenuation of the renal inflammatory response, pathological damage, and serum creatinine [171,172]. The mechanism for potential nephroprotection with vitamin D could be through NF- $\kappa$ B and the renin–angiotensin–aldosterone system (RAS). NF- $\kappa$ B family transcription factors mediate the immune response through regulation of inflammation and fibrosis gene expression including TNF- $\alpha$ , MCP-1, PAI-1, and additional proinflammatory mediators [173]. Renal MCP-1 enhances macrophage infiltration, which releases mediators that increase renal injury severity [174]. In experimental AKI, NF- $\kappa$ B is noted to be activated in the renal tubular cells [175]. In renal transplant patients with delayed graft function due to AKI, NF- $\kappa$ B activation can be demonstrated in kidney biopsy tissue [176]. 1,25(OH) $_2$ D downregulation of the expression of genes for IL-12, IL-8, MCP-1, PAI-1, angiotensinogen, and microRNA-155 by blocking NF- $\kappa$ B activation via VDR physical interaction with IKK $\beta$  [177]. VDR-deficient fibroblasts have constitutional activation of NF- $\kappa$ B [178]. In an experimental model, paricalcitol, a vitamin D analog, which binds VDR, reduces kidney-related inflammation by NF- $\kappa$ B activity blockade [179]. In vitro, endothelial cells derived from human coronary arteries, proinflammatory cytokine production is blunted by 1,25(OH) $_2$ D through interference of the TNF- $\alpha$ -induced activation of NF- $\kappa$ B [180]. In experimental AKI, expression of NF- $\kappa$ B was attenuated with paricalcitol pretreatment [181].

Angiotensin II is well known as a vasoconstrictor that also acts as an inducer of cell proliferation, inflammation, oxidative stress, and fibrogenesis. Angiotensin II-induced reactive oxygen species may have an important role in AKI. During kidney injury, angiotensin II induces profibrotic, proinflammatory cytokines, and growth factors [182]. Angiotensin II is also related to enhancement of immune cell infiltration, extracellular matrix synthesis, podocyte damage, cell hypertrophy, and cell proliferation [182]. Evidence suggests that 1,25(OH) $_2$ D inhibits gene transcription of renin through the cAMP-signaling pathway [183].

#### 5.2.4 Acute lung injury

In community-dwelling patients, self-reported respiratory infection is lower in patients with high 25(OH)D [184]. In outpatients, pneumonia development is noted to be lessened with higher vitamin D levels [185]. Study of hospitalized patients with pneumonia shows that low 25(OH)D levels are associated with heightened mortality and improve the performance of a pneumonia severity index score [186]. Critically ill patients with

preexisting low vitamin D levels have higher risk of development of acute lung (ALI) and a higher mortality if ALI develops [187].

Vitamin D is a robust stimulator of antimicrobial peptides in the lung. 1,25(OH) $_2$ D has the ability to induce the release of the potent proinflammatory protein LL-37 within the lung bronchial epithelium [109]. LL-37 is stored at high concentrations in neutrophil secondary granules in the inactive proform hCAP18. LL-37 is present in airway secretions and induced in response to inflammation and infection [188]. High concentrations of LL-37 are found in bronchial alveolar lavage fluid from ARDS subjects compared with control subjects [189].

In vitro, conversion of 25(OH)D to 1,25(OH) $_2$ D is observed in respiratory epithelial cells [190]. Exposure of respiratory epithelial cells to 25(OH)D results in upregulation of LL-37 and CD14 [190]. 1,25(OH) $_2$ D is reported to decrease the activation level of lung microvascular endothelial cells, with reduction of ICAM-1, ELAM-1, iNOS, and PAF-related neutrophil adhesion [191].

Evidence suggests that in addition to the AMP effects, LL-37 may be involved in epithelial repair in ALI [192]. Further, in vivo experiments with human neutrophils show increased local LL-37 in response to vitamin D increases phagocytosis of bacteria and decreases TLR agonist-mediated increases in TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 [193].

Experimental models in ALI and vitamin D supplementation are not consistent. In a hamster model of ALI, 1,25(OH) $_2$ D inhibits neutrophil recruitment by 40%, postulated to be related to inhibition of IL-8 [194]. In a rat model of ALI associated with ischemia reperfusion of bilateral femoral arteries, 1,25(OH) $_2$ D pretreatment blunts observed lung injury [195]. In a rodent model, 1,25(OH) $_2$ D pretreatment is shown to attenuate seawater aspiration-induced ALI, thought to be due to NF- $\kappa$ B and RhoA/Rho kinase pathway inhibition [196]. In a rodent model of LPS-induced ALI, the absence of VDR was associated with more severe ALI, while vitamin D pretreatment attenuated LPS-induced lung injury with preservation of alveolar barrier function [197]. Others have found no difference in the severity of LPS-induced ALI with regard to vitamin D deficiency [198].

### 5.3 Interventional data

Vitamin D treatment to achieve a sufficiency target (e.g., 25(OH)D > 20 ng/mL) [20] has not yet been established to improve patient outcomes [199]. Vitamin D intervention is an attractive therapeutic target in the critically ill as it is inexpensive, has substantial biological

evidence of efficacy, and appears safe in trials of ICU patients [15,200,201]. Outside the ICU, some interventional trials show an antiinflammatory and immunomodulatory role of vitamin D [202,203]. Some studies on vitamin D supplementation show improvement related to bacterial [203,204] and viral infection outcomes [205]. A systematic review and metaanalysis of 43 RCTs suggests vitamin D supplementation in those with low 25OHD < 12 ng/mL may reduce respiratory infections [206].

It is important to underscore that vitamin D supplementation studies do not consistently show benefit [207–211]. Single high-dose annual vitamin D supplementation in older women was shown to increase fall and fracture risk [212]. In hospitalized children with pneumonia, two vitamin supplementation D trials failed to show decreased illness duration, but one had a lower pneumonia recurrence rate [213,214]. Another vitamin supplementation D trial did not demonstrate differences in pneumonia incidence in infants [215]. Vitamin D supplementation trials in adults have failed to demonstrate differences in respiratory infections [207,216,217]. In a high-dose vitamin D interventional trial on outpatients with COPD, the time to first exacerbation outcome was not significantly different [209]. A post hoc analysis in trial patients with very low baseline vitamin D levels showed that COPD exacerbations were less in the intervention arm suggesting that intervention may need to be targeted to a particular 25(OH)D level [209]. When interpreting vitamin D supplementation trials, it is important to take into consideration the design issues including population studied, baseline 25(OH)D status, DBP levels, sufficient study power, dose given and in what setting, supplementation type (vitamin D<sub>2</sub> vs. D<sub>3</sub> vs. calcitriol), duration of supplementation, severity of illness, case mix, primary outcome, and the event rate of primary outcome.

The Correction of Vitamin D Deficiency in Critically Ill Patients (VITdAL-ICU trial) was a randomized, double-blind, placebo-controlled, single-center trial of 475 ICU patients with 25(OH)D ≤ 20 ng/mL randomized 1:1 with 540,000 IU oral cholecalciferol followed by monthly maintenance doses of 90,000 IU for 5 months versus placebo [15]. The primary outcome was hospital length of stay. By the design, investigators only included patients with low vitamin D levels. The results show that there was not a significant difference for the primary outcome length of stay. In the secondary outcome of mortality, there were nonsignificant differences in survival.

The VITdAL-ICU trial showed a survival benefit in subjects with very low vitamin D (25(OH)D ≤ 12 ng/mL) who were randomized to the vitamin D group [15]. In trial subjects with very low vitamin D (N = 200; 42% of the study population), hazard ratios for mortality among the vitamin D group, as compared

with the placebo group, were as follows: in-hospital mortality HR = 0.56 (95% CI, 0.35–0.90; *P* = .01); 28-day mortality HR = 0.52 (95% CI, 0.30–0.89; *P* = .02), and 6-month mortality HR = 0.60 (95% CI, 0.39–0.93; *P* = .02). In trial subjects with very low vitamin D, the absolute risk reduction was 15.9% for 28-day mortality and the number needed to treat was 6. This interventional study is supportive but not definitive of the hypothesis that vitamin D supplementation targeted to critically ill patients with very low vitamin D [25(OH)D ≤ 12 ng/mL] may improve outcomes [15]. While the study is considered a neutral trial, there is still much progress to be made, the balance of evidence supports vitamin D<sub>3</sub> supplementation as a promising intervention in patients with severe vitamin D deficiency, which merits further investigation.

Though the VITdAL-ICU trial is intriguing, the mortality difference data must be viewed as hypothesis generating as it was not the primary outcome, and the trial was not powered to mortality. The results of the VITdAL-ICU trial should not be used to guide therapy but are helpful in deciding how to design the next large trial on vitamin D supplementation. Paramount issues for trial design in vitamin D are the understanding that for vitamin D supplementation to work, investigators must identify the population that may benefit. The VITdAL-ICU trial may assist investigators with issues regarding sample size, drug, dose, duration, and identification of a subgroup (i.e., 25(OH)D ≤ 12 ng/mL) that may benefit most from supplementation. Further trials are warranted based on the strength of the biological data, the observational data, and the limited interventional data in critically ill patients. If replicated in a multicenter randomized controlled trial, the VITdAL-ICU trial data indicate that the most fruitful time to intervene is early during an ICU course, an appropriate dose of oral cholecalciferol is in the high range, and the subjects who are most likely to benefit are those with low 25(OH)D at the time of enrollment.

The Cholecalciferol Supplementation for Sepsis in the ICU study was a randomized controlled trial of 30 critically ill patients with new-onset severe sepsis or septic shock randomized 1:1:1 to placebo versus 200,000 IU cholecalciferol versus 400,000 IU cholecalciferol [218]. The primary trial goal was to evaluate the change in circulating 25(OH)D levels, bioavailable 25OHD (determined from 25(OH)D, DBP, and albumin levels), and LL-37 expression. Investigators reported that the 400,000 IU cholecalciferol dose significantly and rapidly increased circulating 25(OH)D levels, bioavailable 25(OH)D, and expression of LL-37. Additionally, it was shown that the 400,000 IU cholecalciferol dose significantly reduced systemic levels of IL-1β and IL-6 [218].

Leaf et al. performed a novel randomized controlled trial of 67 critically ill patients with severe sepsis or

septic shock randomized 1:1 to placebo versus 2 µg intravenous calcitriol supplementation. The primary outcome was plasma hCAP-18 protein levels assessed at 24 h. The investigators found that subjects randomized to calcitriol supplementation had increases in 24 h leukocyte mRNA expression of cathelicidin and the anti-inflammatory cytokine IL-10 but not plasma protein levels [211].

Trials in critically ill patients with ARDS suggest that oral high-dose cholecalciferol may improve length of stay and integrity of the alveolar–capillary barrier. The High-Dose Vitamin D in Lung Failure study was a randomized, double-blind, placebo-controlled, single-center trial of 31 ventilator-dependent ICU patients designed to evaluate the efficacy and safety of oral high-dose vitamin D<sub>3</sub> (placebo vs. 250,000 IU vs. 500,000 IU) [219]. Patients treated with vitamin D<sub>3</sub> demonstrated a statistically higher 25(OH)D and cathelicidin (LL-37) levels, shortened hospital length of stay, and nonsignificant decreases in ICU length of stay and duration of mechanical ventilation. Further, in the Vitamin D Open Label Replacement Esophagectomy study (UKCRN ID 11994), investigators show that 200,000 IU oral cholecalciferol supplementation prior to esophagectomy reduced the observed changes of in vivo measurements of alveolar capillary damage seen in vitamin D–deficient patients [220]. Perioperative changes were determined in an in vivo measure of the integrity of the alveolar–capillary barrier, namely extravascular lung water accumulation (extravascular lung water index [EVLWI]), and pulmonary vascular permeability index (PVPI) using a PiCCO<sub>2</sub> catheter. Severe vitamin D deficiency (25-(OH)D<sub>3</sub> <20 nmol/L) was associated with an increased accumulation of extravascular lung water and evidence of increases of PVPI, a marker of alveolar capillary permeability. Patients supplemented with vitamin D prior to esophagectomy had significantly reduced changes in EVLWI and PVPI than placebo-exposed patients.

The Vitamin D to Prevent Lung Injury Following Esophagectomy trial was a multicenter double-blind, randomized, placebo-controlled trial of high-dose vitamin D in 79 patients undergoing esophagectomy [221,222]. The primary outcome was extravascular lung water index at the end of esophagectomy. Despite effective increase in blood 25(OH)D concentrations and decreased changes in postoperative pulmonary vascular permeability index, the trial was neutral for the primary outcome.

RECTIFY was a single-center, randomized, double-blind, placebo-controlled trial of early high-dose enteral vitamin D<sub>3</sub> supplementation in emergently admitted neurocritical care patients with 25(OH)D ≤ 20 ng/mL [223]. 436 patients were to be randomized with the primary outcome being length of hospital stay. The trial

was halted at interim analysis with 274 patients enrolled for medical futility.

VIOLET was a multicenter, randomized, double-blind, placebo-controlled phase III trial of early high-dose enteral vitamin D<sub>3</sub> supplementation in vitamin D–deficient patients at high risk for ARDS and mortality [16]. Three thousand adult subjects with 25(OH)D ≤ 20 ng/mL were to be recruited from the emergency department with intention to admit to ICU, or ICU patients within 48 h of admission. To be enrolled, patients required acute risk factors for ARDS and mortality directly contributing to the need for ICU admission. Subjects were randomized to high-dose enteral vitamin D<sub>3</sub> or placebo. The VIOLET trial did not find a difference in 90-day all-cause mortality. Recruitment of patients in the emergency department resulted in a cohort with mild critical illness. Half of the VIOLET cohort had a SOFA score ≤ 5. The trial was designed to study patients at high risk for ARDS, but a small proportion of the trial subjects had ARDS or developed new ARDS in 7 days [224].

As noted in the VITdAL-ICU trial [15], critically ill patients likely to benefit from high-dose vitamin D are those with a baseline of 25(OH)D ≤ 12 ng/mL. Inclusion of patients with 25(OH)D >12–20 ng/mL biases the VIOLET trial result to the null as these patients may be less likely to benefit. Similar to the VITdAL-ICU trial, in 25% of VIOLET subjects who received high-dose vitamin D, the plasma 25(OH)D was less than 30 ng/mL at day 3, which also biases the trial result to the null [224]. Such lack of response may confound the intervention outcome relationship as there is a systematic difference between those in the intervention group with plasma 25(OH)D < 30 ng/mL versus those with plasma 25(OH)D ≥ 30 ng/mL. The ongoing VITDALIZE study is designed to evaluate the efficacy of high-dose enteral vitamin D<sub>3</sub> in severely vitamin D–deficient population with higher severity of critical illness [225]. The VITDALIZE study will randomize 2400 adult patients with 25(OH)D ≤ 12 ng/mL to high-dose enteral vitamin D<sub>3</sub> or placebo within 72 h after ICU admission.

Trials in critical illness are hampered by the heterogeneity of the population where the effect of treatment can be dampened, thus decreasing the study power [224,226]. In the VITdAL-ICU trial, for example, half of the patients who received high-dose oral vitamin D<sub>3</sub> did not have the expected increase of 25(OH)D in 72 h [15,141]. Such heterogeneity of treatment effect exists even with enriching trials by only enrolling patients with 25(OH)D levels below a specific serum 25(OH)D target [15]. Similar to administering antibiotics to suspected infections of unknown etiology, the identification of patients who may benefit from intervention is currently limited.



Adequate sample size is essential to determine if differences in outcomes between intervention groups are present [227]. Sample size in randomized trials is determined by the estimated outcome proportion in the placebo group relative to the estimated outcome proportion in those who receive the intervention. Table 106.1 shows the sample size ranges required to have the power to detect differences in the primary outcome (28-day mortality) and vitamin D<sub>3</sub> intervention. In trial design, the event rate of the placebo group must be estimated prior to randomization. There is a high likelihood that such a trial will be underpowered for the outcome of interest unless the sample size is quite large. Most existing vitamin D<sub>3</sub> interventional trials are underpowered for mortality [228].

With the use of research tools such as metabolomics, investigators are beginning to determine metabolic phenotypes or endotypes at ICU admission, which have potential to identify patient subgroups who may benefit from specific therapies such as vitamin D<sub>3</sub> intervention [141,229]. Metabolomics allows for a window into how interventions alter metabolism as a whole and differences in the intervention response among patient subgroups [142,230]. In the future, translation of research findings to point-of-care determination of such subgroups may allow practitioners to provide personalized therapeutics.

## 5.4 Vitamin D dose in critical illness

Recommended adequate intake for adults  $\leq$  age 70 is 600 IU/day and 800 IU/day for  $>$  age 70 [231]. These recommendations can result in a large portion of adults becoming vitamin D deficient. In general, sun avoidance behavior combined with a low proportion of time spent outdoors has decreased sun exposure. Too little vitamin D dietary supplementation in the setting of low sun exposure can result in low vitamin D levels [232]. Most critically ill patients have low vitamin D levels [4–11].

Comorbidities, severity of illness, and organ dysfunction likely contribute to the low 25(OH)D levels observed in ICU patients [6].

The dose of vitamin D supplementation in the critically ill required to achieve a normal 25(OH)D is much higher than current adequate intake. In the critically ill, malabsorption from gastrointestinal edema and inflammation limits the effectiveness of oral and enteral formulations. In general, the efficiency of vitamin D supplementation is dependent on gastrointestinal absorption, renal hydroxylation, hepatic chylomicron transport, and liver degradation of 25(OH)D. The response to vitamin D supplementation may be blunted in critical illness due to organ dysfunction (gastrointestinal, renal, hepatic). In addition, an increase in tissue vitamin D requirement in the critically ill results in heightened tissue level conversion of 25(OH)D to active 1,25(OH)<sub>2</sub>D [233]. Healthy adults have a two- to threefold increased response in 25(OH)D levels when supplemented with single high-dose oral cholecalciferol (300,000–600,000 units) compared with the critically ill [234].

From supplementation studies in the critically ill, we know that at least 120,000 IU total oral cholecalciferol delivered over the course of 1 week is needed to increase 25(OH)D in critically ill septic patients [201]. In the VITdAL-ICU and VIOLET trials, one dose of oral 540,000 IU cholecalciferol was effective in increasing the 25(OH)D levels  $>$  20 ng/mL in most patients within 2 days' time [15,16,200]. In the VITdAL-ICU trial and the pilot trial, large, unpredictable interindividual differences in serum 25(OH)D response to the of oral 540,000 IU cholecalciferol were observed [15,200]. Further, in the VITdAL-ICU and VIOLET trials, only half of patients treated with 540,000 IU vitamin D<sub>3</sub> reached serum 25(OH)D levels of 30 ng/mL or higher [15,16]. This response to vitamin D<sub>3</sub> may be evidence of altered gastrointestinal function of critical illness and/or to renal and drug-related alteration of the hepatic CYP2R1 [235].

**TABLE 106.1** Sample size to detect 10%–50% reduction in 28-day mortality with 90% power assuming statistical significance level at 0.05

		Relative percent reduction in 28-day mortality rate in vitamin D <sub>3</sub> intervention group					
28-day mortality rate in placebo group	50%	40%	30%	25%	20%	15%	10%
40.00%	238	378	684	992	1560	2784	6286
35.00%	284	458	832	1208	1904	3412	7726
30.00%	348	560	1026	1496	2364	4246	9646
25.00%	438	708	1300	1900	3008	4748	12334
20.00%	572	928	1710	2504	3974	7170	16368
15.00%	796	1296	2394	3510	5582	10094	23088

200 IU cholecalciferol packaged in a multivitamin preparation is common in standard parenteral and enteral formulations. This dose is far from what is required to normalize vitamin D status rapidly. Rapid normalization of 25(OH)D may be needed to fully benefit from vitamin D sufficiency during critical illness [236]. Though studies on intravenous vitamin D<sub>3</sub> formulation are published in the human and veterinary literature, it is not available commercially. Intravenous vitamin D<sub>3</sub> would be ideal for high-dose supplementation and bypass the gastrointestinal malabsorption and the hepatic first-pass effect common in the ICU population [237]. Further, although there are few studies to support the use of supplemental calcifediol [25(OH)D] or calcitriol [1,25(OH)<sub>2</sub>D] in the ICU, it may be a reasonable approach to quickly increase vitamin D in critically ill patients without efficient liver or kidney hydroxylation of cholecalciferol [211] (see Chapter 73 for more detailed discussion of calcifediol).

### 5.5 Is vitamin D supplementation safe?

The safe tolerable upper intake level (UL) for adults is 10,000 IU/d per the Endocrine Practice Guidelines Committee [21], while the Institute of Medicine has a more conservative approach with an UL of 4000 IU/d [20]. The Office of Dietary Supplements (ODS) recommends a toxicity threshold of serum 25(OH)D levels of 200–240 ng/mL and administration level of 10,000–40,000 IU per day [238]. These recommendations are not made in the context of critical illness, and it is not clear what the ideal dose for vitamin D supplementation should be in the critically ill. As demonstrated in trials, high-dose supplementation is needed to increase 25(OH)D levels in the critically ill. High doses of vitamin D supplementation can lead to vitamin D intoxication, which has the rare potential to be life-threatening.

Vitamin D intoxication is not likely at intakes below 10,000 IU per day, but any elevated dose over time may cause untoward health effects [238]. Toxicity cases are usually related to accidental and protracted consumption of over 40,000 IU per day [239]. Vitamin D toxicity has been described in patients with *CYP24A1* mutations causing failure to metabolize 1,25(OH)<sub>2</sub>D [240]. Important issues with trial design and the potential adoption of high-dose vitamin D in the ICU are the safety profile of high or “supraphysiologic” (relative to current adequate intake) doses of vitamin D and the risk of toxicity. The IU dose of vitamin D needed for toxicity in humans is not known. The lethal dose in 50% (LD50) in rats is 352 mg/kg oral vitamin D<sub>3</sub>, which is equal to 14 million IU/kg, 28 times the dose given in the VITdAL-ICU trial [241]. In general, vitamin D toxicity in the form of hypercalcemia is only seen

when serum 25(OH)D is kept above 150–200 ng/mL [242]. In randomized controlled trials that utilized ≥100,000 IU single-dose oral vitamin D<sub>3</sub>, the adverse events demonstrated have been rare mild hypercalcemia [15,200,201] and the increase in falls/fractures in older community-dwelling women [212].

Studies have shown the safety profile and efficacy in increasing 25(OH)D of single high-dose cholecalciferol administration in rheumatologic and elderly patients [243,244] and in the ICU [15,200]. In an outpatient elderly population of 63 subjects who received an oral 500,000 IU vitamin D<sub>3</sub> loading dose, the mean increase in 25(OH)D was 23 ng/mL without hypercalcemia [243]. Similarly, in a study of rheumatology outpatient population of 33 subjects who received a single loading dose of oral 300,000 IU vitamin D<sub>3</sub>, the mean increase in 25(OH)D was 27 ng/mL after 3 months with two cases of mild hypercalcemia reported [244]. Further, in the pilot study of the VITdAL-ICU trial of 25 medical ICU patients, a single dose of oral 540,000 IU vitamin D<sub>3</sub> normalized 25(OH)D in most patients and showed no hypercalcemia [200]. In the VITdAL-ICU trial of 475 ICU patients, a single dose of oral 540,000 IU vitamin D<sub>3</sub> followed by monthly oral 90,000 IU vitamin D<sub>3</sub> showed no adverse effects other than mild hypercalcemia in a small number of subjects [15]. The 2 highest subject 25(OH)D levels achieved in the VITdAL-ICU trial were much lower than what is considered toxic 25(OH)D [15]. In other high-dose oral vitamin D<sub>3</sub> intervention studies in the ICU [200,201], the peak 25(OH)D level achieved was 64 ng/mL, well below toxic 25(OH)D levels. The small numbers of subjects with adverse effect (mild reversible hypercalcemia) in the high-dose oral vitamin D<sub>3</sub> trials are reassuring but only in the context of the small sample size.

As it appears that high oral doses of vitamin D<sub>3</sub> are required to improve 25(OH)D status in the critically ill, it is important to review the potential for unexpected harm with such a dosing strategy. Specific populations may be very sensitive to vitamin D<sub>3</sub> administration and include those with sarcoidosis and *Mycobacterium* infections, myeloma patients treated with thiazide diuretics, and those with mutations in *CYP24A1* [245]. If efficacy of high-dose vitamin D<sub>3</sub> is established in randomized controlled trials on specific populations and subsequently adopted by the ICU community on a wide scale, surveillance of drug adverse effects will be required to identify populations at risk for adverse events.

Though response to high-dose oral vitamin D<sub>3</sub> in the critically ill is not expected to be similar to outpatient subjects, it is important to consider adverse findings in trials of high-dose vitamin D<sub>3</sub>. In a landmark trial of 2256 community-dwelling women randomized to oral 500,000 IU cholecalciferol or placebo, an increased risk

of falls and fractures was demonstrated in the vitamin D group. The incidence rate for falls was increased by 15% and for fractures by 26% in the vitamin D group [212]. In a small subset with serum available ( $n = 131$ ), the median baseline serum 25(OH)D was 20 ng/mL. Evaluation of bone turnover markers in a study of 12 elderly outpatients administered oral 600,000 IU cholecalciferol compared with 24 control patients. Investigators noted increased C-terminal-telopeptides of type I collagen and cross-linked N-telopeptide of type I collagen (bone resorption markers) within 24 h of supplementation which resolved by 90 days [246]. In the context of the relatively short half-life of cholecalciferol (2–3 weeks) and the increased risk of falls and fractures, a high-dose vitamin D supplementation given once per year may be harmful in this population. The reason for the increase in falls and fracture is not known, but high oral vitamin D<sub>3</sub> may upregulate 25-hydroxyvitamin D-24-hydroxylase, which in turn degrades active vitamin D, thus decreasing tissue availability [247]. If this potential defensive catabolism occurs in the face of high-oral vitamin D<sub>3</sub>, perhaps giving smaller doses with higher frequency (4000 to 10,000 IU) [20,21] could be more efficacious in the ICU.

## 6. Conclusions

Vitamin D in the ICU setting is understudied. It is not known what the acute effects of treatment of vitamin D deficiency in the ICU setting, where multiple drugs (and potential drug interactions) and multiple interventions are going on at the same time, will be. 25(OH)D testing is not routine in the ICU, nor is it recommended in the general population, unless there are clear indications for suspecting vitamin D deficiency [20]. Neither the IOM nor the Endocrine Society addresses the issue of vitamin D deficiency in the ICU setting [21].

Vitamin D supplementation is not recommended routinely for mortality benefits in any situation, given current knowledge. Some recommendations or guidelines for the screening and treatment of vitamin D deficiency in the ICU setting do exist [248]. There are no standard clinical protocols for vitamin D supplementation in the ICU setting. Over the next several years, further interventional trials sufficiently powered to detect differences in outcomes may shed light on the efficacy of vitamin D supplementation in the ICU and specific populations that may benefit. Therefore, treating ICU patients with vitamin D to alter ICU outcomes at any dose at this time remains experimental and not standard of care [16].

## 7. Summary points

- Low vitamin D levels are commonly found in critically ill patients.
- Vitamin D has important roles in the genetic, epigenetic, and metabolomic response to critical illness.
- Benefit of vitamin D supplementation in the critically ill is not consistently shown.

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# Index for Volume 2

Note: 'Page numbers followed by "f" indicate figures and "t" indicates tables and "b" indicate boxes'.

- #
- 1,24,25-trihydroxyvitamin D, 1076
- 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D), 11, 78, 126, 149, 185–186, 223, 248, 265, 304, 352, 412, 589, 594, 603–604, 607–608, 664–665, 720–721, 996–999, 1001–1002, 1011–1012, 1059, 1092, 1185–1186
- and antimicrobial peptides in antiviral immunity, 1017–1018
- and antiviral CD4 T cell immunity, 1019–1021
- and antiviral CD8 T cell immunity, 1021 and antiviral cytokine and chemokine responses, 1018–1019
- and B cell responses to viruses, 1021–1022
- catabolism in chronic kidney disease, 592–594
- corrects impaired 25(OH)D availability to nonrenal 1 $\alpha$ -hydroxylase in CKD, 596–599
- decreased clearance of, 634
- deficiency, 599–600
- role in pathogenesis of secondary hyperparathyroidism in chronic kidney disease, 599f
- 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D)-VDR-activatable enhancer elements, 457
- endogenous production of, 630–634
- evolutionary aspects of 1,25(OH)<sub>2</sub>D induction of AMPs and PRRs, 1000–1001
- feedback, 593
- hereditary resistance to, 314
- induces antimicrobial peptides implicated in antibacterial immunity, 997–998
- induction
- of ACE2 gene expression, 607
- of FGF23 synthesis, 600
- and innate immune sensing of viruses, 1016–1017
- mechanism of 1,25(OH)<sub>2</sub>D action relevant to HVDNR, 364–365
- and natural killer cell responses, 1019
- and nonclassical immune cells, 1002
- 1,25-(OH)<sub>2</sub>D-mediated hypercalcemia, 465–466
- 1,25(OH)<sub>2</sub>D-mediated suppression, 607 of ADAM17 gene, 598
- 1,25(OH)<sub>2</sub>D/VDR, 601
- 1,25(OH)<sub>2</sub>D/VDR suppression of ADAM17 gene, 601
- alterations in 1,25(OH)<sub>2</sub>D/VDR endocrine, 596–599
- complex, 598, 602
- pathogenesis of parathyroid hyperplasia and 1,25(OH)<sub>2</sub>D resistance in CKD, 600f
- production by extrarenal 1 $\alpha$ -Hydroxylases in chronic kidney disease, alterations in, 594–596
- promotes expression of pattern recognition receptors and cytokines, 999–1000
- regulates release of reactive oxygen species, 1000
- regulation of antibacterial innate immunity, in vivo evidence for, 1002–1004
- regulation of gene expression by, 364–365
- replacement therapy, 587
- toxicity, 628–629
- 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), 457, 625, 628–629, 631, 634–637, 652, 679, 682–683, 687–688, 693–696, 702f, 741, 772–774, 798, 904, 918, 968, 1109, 1130, 1153–1155
- and acquired immunity, 766–768
- and analogs and impact of genomic ancestry, chemotherapy of PCA with, 926–927
- clinical use, 1214–1217
- combination of vitamin D analogs with other therapies, 1216–1217
- nail psoriasis treatment, 1216
- psoriatic lesions treatment in HIV patients, 1216
- scalp psoriasis treatment, 1216
- skin lesions treatment in children, 1216
- treatment of face and flexures, 1216
- downregulation of c-MYC expression in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation, 762–763
- effects, 800f–802f, 804f
- genes in human colon carcinoma cells, 872f
- and hematopoiesis, 766
- hyperresponsivity in human osteoclast precursors, overexpression of
- TAF12 is sufficient to induce, 654–655
- and innate immunity, 766
- interact ions, 803f
- interplay between 1,25(OH)<sub>2</sub>D<sub>3</sub> and CD4<sup>+</sup>Tr1 cells in MS, 1149
- 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced hypercalcemia, 633
- marrow stromal cells derived from P62<sup>P394L</sup> knock-in mice and P62<sup>P392L</sup> Paget's patients hyperresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub> and display enhanced RANKL production in response to, 657
- mechanism of action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoclast formation, 652
- mechanism of action of 1,25(OH)<sub>2</sub>D<sub>3</sub> in colorectal cancer, 868–881, 868
- antagonism of Wnt/ $\beta$ -catenin pathway, 868–871
- antiangiogenic, antiinflammatory, immunomodulatory, and detoxifying effects, 874–878
- effects in colon cancer-associated fibroblasts, 878–880
- effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> in colorectal stem cells–derived organoids, 880–881
- effects on colon cancer cell proliferation, survival, and differentiation, 871–874
- target genes in colon cancer cells, 871–872
- mechanisms of resistance toward anticancer actions of, 922–923
- 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated gene expression, 843
- modulation of IL-10 and CD4<sup>+</sup>Tr1 cell production, 1147–1148
- multilevel antagonism of Wnt/ $\beta$ -catenin signaling pathway, 862f
- osteoclast precursors from TRACP–TAF12 transgenic mice hyperresponsive to, 655
- as potential CD4<sup>+</sup>Tr1 cell inducer in MS patients, 1148–1149
- protects against CRC acting on carcinoma cells, cancer-associated fibroblasts, and cancer stem cells, 879f
- regulated genes, 841–842
- regulation of breast cancer stem cell populations, 847–848



- 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>)  
(*Continued*)  
responsivity of OCL precursors from  
Paget's patients, mechanism of  
action of TAF12 in increased,  
655–656  
retinoblastoma protein control of  
1,25(OH)<sub>2</sub>D<sub>3</sub>-induced G1 block, 762  
serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in  
Paget's patients, 659  
supports pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cells  
promote myelin repair and  
replacement, 1150  
supports protective pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg  
cell differentiation, 1138–1139  
in vitro analyses of mechanisms  
mediate 1,25(OH)<sub>2</sub>D<sub>3</sub> sensitivity,  
921  
in vivo analyses of, 921–922
- 25-hydroxyvitamin D (25(OH)D), 11, 32, 47,  
78, 97, 135, 150, 167, 185, 201,  
242–243, 304, 341, 360–361, 411–412,  
443, 489, 511, 567–568, 609, 625, 659,  
665, 681, 949, 1011, 1076, 1103, 1130,  
1259  
alterations in vitamin D bioactivation to  
25(OH)D in chronic kidney disease,  
589–592  
catabolism in chronic kidney disease,  
592–594  
concentrations  
25(OH)D synthesis and metabolism,  
185–186  
applications and practical  
implications, 193–194  
factors affecting, 98–101  
genome-wide association studies of  
25(OH)D, 186–187  
geographic location and ultraviolet B  
doses, 100  
GxE, 192–193  
heritability of serum 25(OH)D levels,  
186  
meteorological factors, 100–101  
seasonal changes in, 98–99, 98t–99t  
thirty-five SNPs, 188t  
travel to sunny locations in winter,  
101  
urban *vs.* rural residence, 101  
variants with evidence for replicated  
association with 25(OH)D, 187–192  
determining optimal serum 25-  
hydroxyvitamin D concentrations  
for musculoskeletal health, 415–416  
endogenous production of, 630  
factors affecting 25-hydroxyvitamin D  
concentrations, 98–101  
behavioral determinants, 102–107  
endogenous characteristics, 101–102  
measures of circulating 25(OH)D, 33–34  
metabolites, 149–150  
and MS, 1170–1171  
prospective studies, 1170f  
vitamin D genes and MS risk,  
1171–1175
- 25(OH)D-1 $\alpha$ -hydroxylase, 589, 620,  
995–996, 1131–1132  
serum 25(OH)D concentration  
thresholds, 118t  
status and disparity of PCa in men of  
African genomic ancestry, 920  
studies using predicted 25(OH)D,  
727–728  
thresholds for, 117–118  
vitamin D and 25(OH)D toxicity,  
625–628
- 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>), 457,  
620, 742, 772–774, 798, 1036  
1 $\alpha$ -hydroxylase, 327, 360–361,  
1185–1186  
redox imbalance, 1151–1152  
25-hydroxyvitamin D<sub>3</sub>–24-hydroxylase,  
372
- A**  
A-ring  
resulting in PRI-5201 and PRI-5202  
analogs, 769  
results in superanalogs, 1118–1119  
of vitamin D metabolite, 664  
AA. *See* African American (AA); Alopecia  
areata (AA)  
 $\alpha$ -SMA. *See*  $\alpha$ -smooth muscle actin ( $\alpha$ -  
SMA)  
 $\beta$ -TrCP. *See*  $\beta$ -transducin repeat-containing  
protein ( $\beta$ -TrCP)  
AACE. *See* American Association of  
Clinical Endocrinologists (AACE)  
AAP-CON. *See* American Academy of  
Pediatrics Committee on Nutrition  
(AAP-CON)  
AAT. *See* Alpha-1 antitrypsin (AAT)  
ABC transporters. *See* ATP-binding  
cassette transporters (ABC  
transporters)  
ABCA1 gene, 187–189  
Aberrantly differentiated endocrine  
exocrine (ADEX), 951–952  
aBMD. *See* Areal bone mineral density  
(aBMD)  
Absolute risk reduction (ARR), 427  
“Absorptive” hypercalciuria, 620  
Accelerated skeletal mineralization,  
hypocalcemia due to, 315  
Accessory proteins, 282  
ACE. *See* American College of  
Endocrinology (ACE); Angiotensin  
II–converting enzyme (ACE);  
Angiotensin-converting enzyme  
(ACE)  
ACE2. *See* Angiotensin-converting  
enzyme II (ACE2)  
Acetyl-CoA carboxylase inhibitors, 560  
Acid isomerization of vitamin D<sub>3</sub>, 80  
Acidic, serine, and aspartic acid-rich  
motif (ASARM), 286–287  
Acitretin, 1216–1217  
Acne, 1222–1223  
Acquired autoimmune disorder, 310  
Acquired disorders, 306  
Acquired hypophosphatemic disorders  
with elevated FGF23 levels, 293.  
*See also* Genetic hypophosphatemic  
syndromes of excess FGF23  
TIO, 293  
Acquired immunity, 1,25(OH)<sub>2</sub>D<sub>3</sub> and,  
766–768  
Acquired immunodeficiency syndrome  
(AIDS), 1012  
Acquired vitamin D disorders, 666–667  
ACS. *See* American Cancer Society (ACS)  
ACTE1P. *See* Actin Epsilon 1, Pseudogene  
(ACTE1P)  
Actin Epsilon 1, Pseudogene (ACTE1P),  
191  
Actinic keratosis (AK), 1210–1211, 1222  
Action spectra for vitamin D synthesis  
and skin cancer, similarity of, 968  
Activate protein kinase A (PKA), 282  
Activated T cells, 1076  
Activation-induced cell death, 1117–1118  
Activator protein-1 (AP-1), 745–746  
transcription factor, 753–754  
Active disease, 1078–1079  
Active endocrine organ, 171  
Active hormonal vitamin D [1,25(OH)<sub>2</sub>D],  
489  
Active metabolite, 126  
Active process, 1076  
Active rickets, biomarkers of, 249–250  
Active tuberculosis, 466–467, 1076  
Active vitamin D, 1043  
analogs on fractures, effects of, 427  
diabetes prevention with active vitamin  
D study, 538–539  
metabolites, 411–412  
Actual sunlight exposure, 48  
Acute exacerbations of COPD (AECOPD),  
1235, 1247–1248  
Acute granulomatous pneumonitis,  
632–633  
Acute hepatitis, 1013  
Acute hypophosphatemia, 284  
Acute illness  
interventional data, 1267–1270  
observational data, 1266–1267  
acute kidney injury, 1266–1267  
acute lung injury, 1267  
sepsis, 1266  
vitamin D and critical illness  
outcomes, 1266  
prevalence of vitamin D deficiency in  
critically ill patient, 1260  
vitamin D and critical care patients,  
1265–1272  
assays for vitamin D, 1265–1266  
vitamin D deficiency risk factors in  
critically ill, 1260–1261  
chronic disease, 1261  
ethnicity, 1260  
medication usage, 1261  
seasonality and sun exposure,  
1260–1261  
severity of critical illness and organ  
failure, 1261

- vitamin D dose in critical illness, 1270–1271  
 vitamin D mechanisms in critical illness, 1262–1265  
   antimicrobial peptides, 1263–1264  
   metabolomics, transcriptomics, and epigenetics of vitamin D, 1264–1265  
   vitamin D supplementation safe, 1271–1272  
 Acute infections, 116–117  
 Acute kidney injury (AKI), 604–605, 1265–1267  
 Acute lung injury (ALI), 1267  
 Acute management of hypocalcemia, 316 adults, 316  
   newborns and children, 316  
   role of magnesium supplementation, 316  
 Acute myeloid leukemia cells (AML cells), 741  
 Acute promyelocytic leukemia (APL), 754  
 Acute respiratory distress syndrome (ARDS), 503, 1014–1015, 1091, 1148  
 Acute respiratory infections (ARIs), 22, 466, 1094, 1236–1240, 1249  
   interventional studies on ARI, 1238–1240  
   observational studies on ARI, including childhood wheezing, 1236–1238  
   potential mechanisms, 1248  
 Acute respiratory pathogens, 1236  
 Acute respiratory syndrome coronavirus (SARS-CoV-2), 284–285  
 AD. *See* Alzheimer's disease (AD); Androgen deprivation therapy (AD)  
 Adalimumab, 1067–1068  
 ADAM17, 600–601, 606  
 Adaptive immune responses, calcifediol treatment of microbial disease to boost human innate and, 465–469  
 Adaptive immune system, 1092  
 Adaptive immunity–vitamin D, translational aspects of vitamin D regulation of, 1045  
 Adaptive immunology, translational studies as window to study effects of vitamin D on, 1036–1037  
 ADCC. *See* Antibody-dependent cell-mediated cytotoxicity (ADCC)  
 ADCY10. *See* Soluble adenylylase cyclase 10 (ADCY10)  
 Adenine (A), 969  
 Adenocarcinoma of lung (LUAD), 902  
 Adenoma. *See* Colorectal cancer (CRC)  
 Adenomatous polyposis coli (APC), 805–807, 860  
   actions of vitamin D on immune microenvironment and function of, 1043–1044  
   effects of vitamin D upon immunometabolism in, 1044–1045  
   mutation, 938  
 Adenovirus, 1014  
 Adenylylase cyclase, 311  
 Adequate intakes (AIs), 3, 5  
 Adequate phosphate, 284  
 ADEX. *See* Aberrantly differentiated endocrine exocrine (ADEX)  
 ADH1. *See* Autosomal dominant hypocalcemia type 1 (ADH1)  
 ADHD. *See* Attention-deficit/hyperactivity disorder (ADHD)  
 Adherens junctions (AJ), 861, 1094  
 ADHR. *See* Autosomal dominant hypophosphatemic rickets (ADHR)  
 Adipokines, 171–172, 499  
   adiponectin, 172  
   leptin, 171–172  
 Adiponectin, 171–172  
 Adipose tissue, 171–172, 513–514, 1261  
   macrophages, 499  
   UCP-1 expression, 496–497  
   as vitamin D reserve, 490–491  
 Adipose-specific VDR-KO, 497–498  
 Adiposity, 489, 491, 493  
 Adjustable gastric band, 163, 165  
 Adolescents, 135–136, 237  
 Adult breast cancer, 36–37  
 Adult germ-free mice, 1060  
 Adult patients with VDDR1A, 333  
 Adult populations in North America and Europe and compare against dietary targets, current intakes of vitamin D in, 138–143  
 Adult T cell lymphoma/leukemia (ATLL), 634  
 Adult vitamin D deficiency. *See also* Vitamin D deficiency  
   vitamin D and falls, 436–438  
   dose frequency, 437  
   dose size, 437  
   potential physiologic basis for  
     adverse effect of bolus doses and high daily doses of vitamin D, 438  
     safety signals, 437  
     study population 25(OH)D levels, 436–437, 437f  
   vitamin D and fracture risk, 438–440  
 Adulthood phenotype, 404  
 Adults, 237, 316  
 Advanced lung cancer, vitamin D in management of, 905–909  
 Adverse vitamin D effects on cardiovascular system, 579–580  
   effect of vitamin D supplementation of 4000 IU daily on risk of hypercalcemia, 579f  
 AECOPD. *See* Acute exacerbations of COPD (AECOPD)  
 AECOPD frequency, 1249  
 Aerosols, 100–101  
 Africa, vitamin D status in, 58, 64t–65t  
 African American (AA), 918  
 African genomic ancestry, 25-Hydroxyvitamin D status and disparity of PCa in men of, 920  
 After Breast Cancer Pooling project, 728  
 AGA. *See* Androgenetic alopecia (AGA)  
*Agaricus bisporus*. *See* White button mushroom (*Agaricus bisporus*)  
 Age, effect of, 101  
 Age-related macular degeneration, 2  
 AHO. *See* Albright's hereditary osteodystrophy (AHO)  
 AHR. *See* Aryl hydrocarbon receptor (AHR)  
 AIBD. *See* Autoimmune bullous diseases (AIBD)  
 AIDS. *See* Acquired immunodeficiency syndrome (AIDS)  
 Air pollution, 100–101  
 AIRE gene, 310  
 Airway disease by vitamin D, protection from exacerbation of, 1048  
 Airway surface liquid (ASL), 1002–1003  
 AIs. *See* Adequate intakes (AIs)  
 AIX. *See* Augmentation index (AIX)  
 AJ. *See* Adherens junctions (AJ)  
 AK. *See* Actinic keratosis (AK)  
 AKI. *See* Acute kidney injury (AKI)  
 AKT pathway, 747–749  
 Albright's hereditary osteodystrophy (AHO), 311  
 Albumin, 1262  
 Albuminuria, 578  
 Aldehyde dehydrogenase 1 (ALDH1+), 847  
 Aldehyde dehydrogenase 1 A1 (ALDH1A1), 869  
 Aldehyde dehydrogenase 1 family member A2 (ALDH1A2), 192  
 ALDH1+. *See* Aldehyde dehydrogenase 1 (ALDH1+)  
 ALDH1A1. *See* Aldehyde dehydrogenase 1 A1 (ALDH1A1)  
 ALDH1A2. *See* Aldehyde dehydrogenase 1 family member A2 (ALDH1A2)  
 Alendronate, 426  
 Alfacalcidol, 480, 630, 683, 768, 774  
 Algal supplements of vitamin D, 125  
 ALI. *See* Acute lung injury (ALI)  
 Alimentary limb, 162  
 ALK. *See* Anaplastic lymphoma kinase (ALK)  
 Alk Phos. *See* Alkaline phosphatase activity (Alk Phos)  
 Alkaline phosphatase (ALP), 223, 249, 256, 264  
 Alkaline phosphatase activity (Alk Phos), 361–363  
 Alkalosis, 303–304  
 All-trans retinoic acid (ATRA), 745–746  
 Allergens, 1035–1036  
 Allergic bronchopulmonary aspergillosis, 1039  
 Allergic diseases, 1232  
 Allergic rhinitis, 216–217  
 Allergies, 1250  
 Allograft rejection, Vitamin D role in, 475–476  
 Alopecia, 364, 387–388  
   in children with HVDRR, 362f  
   HVDRR and, 387–388

- Alopecia areata (AA), 1220–1221  
 ALP. *See* Alkaline phosphatase (ALP)  
 Alpha cells, 1111–1112  
 Alpha-1 antitrypsin (AAT), 1041  
 Altered phosphate load, disorders of, 294–295  
   gastrointestinal malabsorption, 295  
   phosphate deprivation, 294–295  
 Aluminum, 295  
 Alveolar macrophages, 466  
 Alveolartype II cells (AT II cells), 1235  
 Alzheimer's disease (AD), 217. *See also*  
   Behcet's disease; Paget's disease (PD)  
 Ambient UVR, 947  
 AMDHD1 gene, 213–215  
 American Academy of Dermatology, 103  
 American Academy of Pediatrics  
   Committee on Nutrition (AAP-CON), 264  
 American Association for Study of Liver Disease, 480  
 American Association of Clinical Endocrinologists (AACE), 175–176, 446–447  
 American Cancer Society (ACS), 917  
 American College of Endocrinology (ACE), 175–176  
 American Dermatology Association website, 12–13  
 American Diabetes Association glycemic criteria, 542  
 American Society for Metabolic and Bariatric Surgery (ASMBS), 175–176  
 American Thoracic Society (ATS), 1233  
 $\alpha$ -amino acids, 243  
 2-amino-3-methylimidazo[4,5-f] quinoline (IQ), 876–877  
 2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-f]quinoline-7-one (7-OH-IQ), 876–877  
 Aminoquinolones, 641  
 5-Aminosalicylic acid (5-ASA), 1067  
 AML cells. *See* Acute myeloid leukemia cells (AML cells)  
 AML-M3. *See* APL  
 AML1. *See* Runt-related transcription factor 1 (RUNX1)  
 AMPs, evolutionary aspects of  
   1,25(OH)<sub>2</sub>D induction of, 1000–1001  
 AMPs. *See* Antibacterial peptides (AMPs); Antimicrobial peptides (AMPs)  
 Amyotrophic lateral sclerosis, 217  
 Anabolic vitamin D analogs, 427  
 Analogs  
   anabolic vitamin D analogs, 427  
   effects of vitamin D and analogs in preclinical models of prostate, 920–923  
   and impact of genomic ancestry, chemotherapy of PCa with 1,25(OH)<sub>2</sub>D<sub>3</sub> and, 926–927  
 Analytic epidemiologic studies, 39  
 Analytical methodology of vitamin D, 81–82  
 Anaphase promoting complex (APC), 757–758  
 Anaplastic lymphoma kinase (ALK), 902–903, 907  
 Ancillary studies, 453  
 Androgen deprivation therapy (AD), 917  
 Androgen receptor (AR), 697–698, 837–838, 921, 1135  
 Androgenetic alopecia (AGA), 1220  
 Androgenindifferent states, 917  
 Androgens, 921  
   support protective pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cell differentiation, 1139–1140  
 Angina pectoris, 577–578  
 Angiogenesis, 686–687, 874  
*Angiostrongylus vasorum* infections, 667  
 Angiotensin 1-receptor antagonists, 575  
 Angiotensin II type I receptor (AT1R), 1092  
 Angiotensin II–converting enzyme (ACE), 606–607  
 Angiotensin receptor 1 (AT1R), 606  
 Angiotensin-converting enzyme (ACE), 390  
   inhibitors, 575  
 Angiotensin-converting enzyme II (ACE2), 606, 1091–1092  
 Animal models, 881–885  
   chemically-induced colon/colorectal cancer models, 881–882  
   diet studies, 881–882  
   for disease, 1109  
   genetic models of colon/colorectal cancer, 884–885  
   of T1D, 1119  
   vitamin D in food from, 82–84  
   vitamin D signaling impact in animal models of breast cancer, 839–841  
   dietary vitamin D effect, 840–841  
   efficacy of vitamin D compounds to inhibit chemically-induced carcinogenesis, 839  
   effect of VDR ablation on mammary carcinogenesis, 841  
   VDR agonists, diet, and UVR in tumor graft models, 839–840  
   in vivo studies, 839  
 Wnt/ $\beta$ -catenin antagonism by  
   1,25(OH)<sub>2</sub>D<sub>3</sub> in animal models and clinical trials, 870–871  
   xenografted mice, 883–884  
 Animal studies, 834t–836t, 1113–1115  
 Ankylosing spondylitis, 216  
 Antagonism of Wnt/ $\beta$ -catenin pathway by 1,25(OH)<sub>2</sub>D<sub>3</sub> in colon cancer, 868–871  
 Anti-IL6 therapies, 1095–1099  
 Antiangiogenic effects, 874–878  
 Antiapoptotic genes, 805  
 Antibacterial immunity  
   1,25(OH)<sub>2</sub>D  
     and autophagy, 998–999  
     barrier integrity, and complement activation, 996–997  
     evolutionary aspects of 1,25(OH)<sub>2</sub>D induction of AMPs and PRRs, 1000–1001  
     induces antimicrobial peptides implicated in, 997–998  
     and natural killer cells, 1002  
     and neutrophils, 1001–1002  
     and nonclassical immune cells, 1002  
     promotes expression of pattern recognition receptors and cytokines, 999–1000  
     regulates release of reactive oxygen species, 1000  
     in vivo evidence for 1,25(OH)<sub>2</sub>D regulation of, 1002–1004  
 Antibacterial peptides (AMPs), 997  
 Antibiotic resistance, 1068  
 Antibody-dependent cell-mediated cytotoxicity (ADCC), 1021–1022  
 Anticonvulsants, 352  
 Antidiuretic hormone, 311  
 Antigen-presenting cells (APCs), 1020f, 1035, 1092, 1185–1186, 1208  
 Antiinflammatory  
   agents, 771  
   cytokine, 1146  
   effects, 874–878  
   effects, 1185–1189  
   vitamin D/VDR signaling and antiinflammatory responses in liver, 561–562  
 Antimicrobial, effects of vitamin D, 799–801  
 Antimicrobial peptides (AMPs), 1015, 1263–1264  
   1,25(OH)<sub>2</sub>D and antimicrobial peptides in antiviral immunity, 1017–1018  
    $\beta$ -2, 1263  
   cathelicidin, 1263–1264  
   inflammasome activation, 1264  
   production, 1262  
 Antioxidants, 681  
 Antiproliferative properties, 772  
 Antirachitic compounds vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in crystal form and dissolved in organic solvents, 78–79  
 Antiresorptive therapy, 177  
 Antiretroviral therapy, 1012  
 Antisense measles virus nucleocapsid protein construct into osteoclast precursors from patients with Paget's disease reduced expression of TAF12 transduction of, 654  
 Antisense MVNP construct (AS-MVNP construct), 654  
 Antisense construct (AS-CONT), 654–655  
 Antituberculosis therapy, 632  
 Antitumor effects, 691  
   of 1,25(OH)<sub>2</sub>D, 828–829  
 Antiviral CD4 T cell immunity, 1,25(OH)<sub>2</sub>D and, 1019–1021

- Antiviral cytokine and chemokine responses, 1,25(OH)<sub>2</sub>D and, 1018–1019
- Antiviral immunity
- association between vitamin D
    - insufficiency and viral disease in epidemiological and interventional studies, 1012–1015
  - acquired immunodeficiency syndrome, 1012
  - COVID-19, 1014–1015
  - dengue fever, 1013–1014
  - herpes, 1013
  - human cytomegalovirus, 1013
  - mononucleosis, 1013
  - pulmonary diseases, 1014
  - viral diarrhea, 1014
  - viral hepatitis, 1012–1013
  - virus-induced cervical cancer, 1014
- immunological mechanisms of viral control, 1015
- 1,25(OH)<sub>2</sub>D
- and antimicrobial peptides in antiviral immunity, 1017–1018
  - and antiviral CD4 T cell immunity, 1019–1021
  - and antiviral CD8 T cell immunity, 1021
  - and antiviral cytokine and chemokine responses, 1018–1019
  - and B cell responses to viruses, 1021–1022
  - and innate immune sensing of viruses, 1016–1017
  - and natural killer cell responses, 1019
  - schematic of immune network involved in, 1016f
  - vitamin D and immunomodulation, 1015–1016
- ANZ gene. *See* Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZ gene)
- AOM. *See* Azoxymethane (AOM)
- AP site. *See* Apyrimidinic site (AP site)
- AP-1. *See* Activator protein-1 (AP-1)
- ApaI*, 1082–1083
- APC. *See* Adenomatous polyposis coli (APC); Anaphase promoting complex (APC)
- APCs. *See* Antigen-presenting cells (APCs)
- APECED. *See* Autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED)
- APL. *See* Acute promyelocytic leukemia (APL); Atrichia with papular lesions (APL)
- APOB* gene, 187–189
- APOC1* gene, 187–189
- APOE* gene, 187–189
- Apoptosis, 691, 693–695, 1111–1112
- Apparent retention (AR), 87–88
- Apurinic site, 971–972
- Apyrimidinic site (AP site), 971–972
- AR. *See* Androgen receptor (AR); Apparent retention (AR)
- AR response element (ARE), 1143
- AraC. *See* 1-β-d-arabinofuranosyl cytosine (AraC)
- Arachidonic acid, 192
- ARDS. *See* Acute respiratory distress syndrome (ARDS)
- ARE. *See* AR response element (ARE)
- Area under curve (AUC), 173
- Areal bone mineral density (aBMD), 447
- placebo on aBMD in general population, 447–448
- Arg158Cys. *See* Arginine to cysteine (Arg158Cys)
- Arg274His mutation, 377
- Arg274Leu mutant, 377
- Arg343Cys. *See* Arginine to cysteine at amino acid 343 (Arg343Cys)
- Arg391Cys. *See* Arginine to cysteine at amino acid 391 (Arg391Cys)
- Arg391His mutation, 378–379
- Arg73Gln mutation, 373–374
- Arg80Gln. *See* Arginine to glutamine at amino acid 80 (Arg80Gln)
- Arg80Gln mutation, 383–384
- Arginine to cysteine (Arg158Cys), 380
- Arginine to cysteine at amino acid 343 (Arg343Cys), 379
- Arginine to cysteine at amino acid 391 (Arg391Cys), 378
- Arginine to glutamine at amino acid 80 (Arg80Gln), 374, 380
- ARI including COVID-19, use of IRC and ERC in, 467–469
- ARIs. *See* Acute respiratory infections (ARIs)
- Aromatase, 697
- Aromatase gene expression, 840
- ARR. *See* Absolute risk reduction (ARR)
- Array technology, 981
- Arrhythmias, 577–578
- Arteriosclerosis, 577–578
- Arteriosclerosis, 603
- Artificial UV-B, 32
- Aryl hydrocarbon receptor (AHR), 560, 1210
- AS-CONT. *See* Antisense construct (AS-CONT)
- AS-MVNP construct. *See* Antisense MVNP construct (AS-MVNP construct)
- ASARM. *See* Acidic, serine, and aspartic acid-rich motif (ASARM)
- Ascorbic acid, 959
- Asia, vitamin D status in, 55, 61t–63t
- ASK1 kinase, 765
- ASL. *See* Airway surface liquid (ASL)
- ASMBS. *See* American Society for Metabolic and Bariatric Surgery (ASMBS)
- Asn276Tyr. *See* Asparagine to tyrosine at amino acid 276 (Asn276Tyr)
- Asparagine to tyrosine at amino acid 276 (Asn276Tyr), 380
- Assay for transposase-accessible chromatin sequencing (ATAC-seq), 881
- Associated adult Tcell lymphoma/leukemia (ATLL), 633
- Asthma, 797, 1233–1234, 1240–1245, 1249
- control, including exacerbations, 1243–1245
  - asthma exacerbations, 1244–1245
  - responsiveness to corticosteroids, 1243–1244
  - control, including exacerbations, 1249
  - in HVDRR patients, 389–390
  - management, 1244–1245
  - pathogenesis, 1240–1243, 1249
  - potential mechanisms, 1248
- AT II cells. *See* Alveolartype II cells (AT II cells)
- AT1R. *See* Angiotensin II type I receptor (AT1R); Angiotensin receptor 1 (AT1R)
- ATAC-seq. *See* Assay for transposase-accessible chromatin sequencing (ATAC-seq)
- Atezolizumab, 903
- ATF7, TAF12 interacts with, 655–656
- Atherosclerosis, 499
- Athletic performance, 435
- Atlantic salmon (*Salmo salar*), 82, 84
- ATLL. *See* Adult T cell lymphoma/leukemia (ATLL); Associated adult Tcell lymphoma/leukemia (ATLL)
- ATM mutation, 938
- Atmospheric and environmental determinants, 98
- geographic location and ultraviolet B doses, 100
  - meteorological factors, 100–101
  - travel to sunny locations in winter, 101
  - urban *vs.* rural residence, 101
- Atmospheric molecules, 102
- Atopic dermatitis, 670
- Atopic disorders, 1250
- Atopy, 1241–1242, 1250
- ATP-binding cassette transporters (ABC transporters), 701
- ATRA. *See* All-trans retinoic acid (ATRA)
- Atrichia, 364
- Atrichia with papular lesions (APL), 364, 387
- “Atrophic” form of disease, 252
- ATS. *See* American Thoracic Society (ATS)
- Attention-deficit/hyperactivity disorder (ADHD), 217
- AUC. *See* Area under curve (AUC)
- Augmentation index (AIx), 575–576
- Australia, vitamin D status and prevalence of vitamin D deficiency in, 67t–68t
- Australia and New Zealand Multiple Sclerosis Genetics Consortium (ANZ gene), 1172–1174
- Autocrine/paracrine actions in CKD, 596–599



- Autoimmune bullous diseases (AIBD), 1218  
     experimental therapies, 1219–1220  
     vitamin D status in, 1219
- Autoimmune demyelinating disease, 1130
- Autoimmune disease, 389, 667
- Autoimmune disorder, 1109
- Autoimmune polyglandular syndrome type 1, 310
- Autoimmune-polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED), 310
- Autophagolysosome, 1264
- Autophagy, 693–695, 765, 998–999, 1264
- Autosomal dominant hypocalcemia type 1 (ADH1), 310
- Autosomal dominant hypophosphatemic rickets (ADHR), 282–283, 285t, 290–291
- Axin2, 805–806
- Axonal degeneration, 1130
- Azothioprine, 1067
- Azoxymethane (AOM), 807, 874
- B**
- B cell chronic lymphocytic leukemia (B-CLL), 693
- B cells, 801, 1012, 1015–1016, 1189  
     1,25(OH)<sub>2</sub>D and B cell responses to viruses, 1021–1022
- B lymphocytes, 767–768, 1035–1036  
     actions of vitamin D on, 1043
- B-6, 959
- B-cell lymphoma, 466
- B-CLL. *See* B cell chronic lymphocytic leukemia (B-CLL)
- B-ring, 78
- Bacille Calmette Guérin (BCG), 1075
- Bacterial exposure, 1059
- Bacterial flagellin, 995
- Bacterial ligands, 1262
- Bacteroides*, 1060
- BAK, 693
- BAL. *See* Bronchoalveolar lavage (BAL)
- BAME. *See* Black, Asian, or minority ethnicity (BAME)
- Barakat syndrome, 309
- Bariatric surgery, 161–162, 171–172  
     bariatric surgery procedures, 162–163, 163f  
     adjustable gastric band, 163  
     biliopancreatic diversion with duodenal switch, 163  
     roux-en-Y gastric bypass, 162  
     sleeve gastrectomy, 163  
     effective treatment for obesity, 161–162  
     indications for, 162  
     management of osteoporosis after, 177  
     skeletal effects of, 163–166
- Baricitinib, 1095–1099
- Barrier integrity, 996–997, 1011–1012
- Barrier tissues, 1263
- Basal cell carcinoma (BCC), 967, 1210–1211
- Base excision repair (BER), 971–972
- Base pairs, 376
- Baseline serum, 416
- Baseline vitamin D, 1239
- Basic leucine zipper ATF-like transcription factor 2(Batf2), 752–753
- Basic structural units (BSU), 229
- Batf2. *See* Basic leucine zipper ATF-like transcription factor 2(Batf2)
- BAX, 693
- BB rat. *See* Biobreeding rat (BB rat)
- BCa. *See* Breast cancer (BCa)
- BCAA. *See* Branched-chain amino acid (BCAA)
- BCAS1. *See* Brain-enriched myelin-associated protein 1 (BCAS1)
- BCC. *See* Basal cell carcinoma (BCC)
- BCG. *See* Bacille Calmette Guérin (BCG)
- BCR. *See* Cell antigen receptor (BCR)
- Beclin-1, 998–999
- Bedouin, 292–293
- Beef, 86
- Behavioral approach, 407
- Behavioral determinants, 102–107  
     lack of knowledge of benefits of vitamin D, 104–105  
     lifestyle factors associated with reduced sun exposure, 105  
     messages to get UV exposure mornings and afternoons, 102–103  
     migration/migrants, 105–107  
     occupation, 103–104  
     outdoor recreational activities, 104  
     shade, 102  
     sun avoidance because of concern about skin cancer, melanoma, 103  
     use of sunscreen, 102
- Behcet's disease, 217. *See also* Alzheimer's disease
- "Beige" adipocytes, 494
- BER. *See* Base excision repair (BER)
- Beta cells, 1109, 1112  
     function, 512–513, 526–532, 543–544  
     vitamin D and, 1111–1113
- Beta-carotene, 16
- Betaine, 1153–1155
- Betaine–homocysteine methyltransferase (BHMT), 1130–1131, 1152–1155, 1153f
- BHMT. *See* Betaine–homocysteine methyltransferase (BHMT)
- Bhmt* gene, 1152, 1154
- Biallelic CYP24A1 mutations, 404
- Biases reduced or eliminated to extent possible, 37
- Bidirectional Mendelian randomization data analysis (Bidirectional MR data analysis), 491
- Bidirectional MR data analysis. *See* Bidirectional Mendelian randomization data analysis (Bidirectional MR data analysis)
- "Big Bang" model, 860
- Bile acid, 352, 466–467
- Biliopancreatic diversion (BPD), 162–163, 295  
     with duodenal switch, 163, 165
- Bioaddition, 150
- Biobreeding rat (BB rat), 1113
- Biochemical  
     abnormalities, 248–249  
     vitamin D effects on biochemical risk markers, 574–575
- Biofortification, 150  
     strategy, 84–85
- Biofortified foods, 151–153
- Biofortified meats, 151–153
- Biologics, 1067–1068
- Bisphosphonates (BPs), 405, 640, 659, 687
- Black, Asian, or minority ethnicity (BAME), 1095
- Black Box, 318
- Bladder cancer, 190
- Blood 25(OH)D levels, optimizing, 543–545
- Blood samples, 40, 1095
- BMD. *See* Bone mineral density (BMD)
- BMDD. *See* Bone mineral density distribution (BMDD)
- BMI. *See* Body mass index (BMI)
- BMP. *See* Bone morphogenic protein (BMP)
- Body mass index (BMI), 101, 161, 162t, 187–189, 201, 428, 462, 489, 545, 827, 950, 1095  
     effect modification of vitamin D supplementation by, 545–546
- Body surface area (BSA), 97–98
- Body's immune system, 1058
- Boletus edulis*, 84
- Bone, 2, 171–172, 226  
     biopsy, 223  
     in CKD patients, 602–603  
     bone formation effects of ghrelin, 173–174  
     deformities, 248  
     deleting TAF12 in mouse OCLs inhibits bone destruction, 657–658  
     density, 252  
     RCTs of effects of vitamin D supplementation on, 447–448  
     health, 117–118  
     current recommended intakes based on bone health goals, 446–447  
     and osteoporosis foundation, 446–447  
     histomorphometry, 228  
     in osteomalacia, 227–228  
     loss  
         adipokines, 171–172  
         bone marrow adipose tissue, 175  
         calcium, 168–170  
         gut-derived hormones, 172–175  
         mechanical unloading, 170–171  
         of muscle mass, 171  
         nutritional deficiencies, 167–170  
         potential mechanisms of, 166–175, 167t  
         sex steroids, 172  
         vitamin D, 167–168

- marrow adipose tissue, 175  
 mass, 163–166  
   adjustable gastric band, 165  
   biliopancreatic diversion with duodenal switch, 165  
   Roux-en-Y gastric bypass, 164  
   sleeve gastrectomy, 164–165  
 matrix, 225  
 microarchitecture, 229  
 microstructure, 165  
 pain, 292  
 remodelling, 315  
 resorption, 687  
 strength, 228–229  
 terminology related to bone formation and mineralization, 224, 224t  
 turnover, 163–164, 249  
   markers, 164  
   volume, 412  
 Bone mineral density (BMD), 128, 164, 215–216, 228–229, 252, 329, 386, 411–412, 438, 443–446, 476–477, 1061  
   vitamin D bone mineral density and falls on, 416–418  
 Bone mineral density distribution (BMDD), 228–229  
   in osteomalacia, 227–228  
 Bone morphogenic protein (BMP), 288  
 Bone-remodeling, deleting TAF12 in mouse OCLs inhibits, 657–658  
 Bookmarking functions, 925  
*Bordetella bronchiseptica*, 1263–1264  
 Bowen's disease, 1210–1211  
 BP. *See* Bullous pemphigoid (BP)  
 BPC3. *See* Breast and Prostate Cancer Cohort Consortium (BPC3)  
 BPD. *See* Biliopancreatic diversion (BPD)  
 BPD with duodenal switch (BPD-DS), 163  
 BPD-DS. *See* BPD with duodenal switch (BPD-DS)  
 BPs. *See* Bisphosphonates (BPs)  
 BRAF, 902–903  
 BRAF. *See* v-Raf murine sarcoma viral oncogene homolog B (BRAF)  
 Brain-enriched myelin-associated protein 1 (BCAS1), 192  
 Branched-chain amino acid (BCAA), 998–999  
 BRCA1 mutation, 938  
 BRCA2 mutation, 938  
 Bread and alternative vitamin D products, 86–87  
 Breast and Prostate Cancer Cohort Consortium (BPC3), 731  
 Breast cancer (BCa), 721–722, 726, 810, 825.  
   *See also* Skin cancer  
   breast cancer cells of origin, 827  
   deregulation of vitamin D pathway in breast cancer, 837–838  
   mechanistic insight into vitamin D actions, 844–848  
   cell metabolism, 846–847  
   regulation of breast cancer stem cell populations, 847–848  
   molecular heterogeneity of human breast cancer, 826  
   observational and interventional studies, 827–832, 827t–828t  
   and breast cancer subtypes, 830  
   population studies, 827  
   vitamin D supplementation trials in breast cancer prevention, 831–832  
   risk, 828–829  
   subtypes and prognosis, 825–826  
 Breast epithelial cells, 810  
 Breast milk, 312–313  
   feeds, 263  
 Breg. *See* Regulatory B cells (Breg)  
 Bronchial epithelial cells, 902  
 Bronchiolitis, 1094, 1232  
 Bronchoalveolar lavage (BAL), 1038  
 Brown adipose tissue, 496–497  
 BSA. *See* Body surface area (BSA)  
 BSC2 gene, 378  
*BsmI*, 1082–1083  
 BSU. *See* Basic structural units (BSU)  
 BTG antiproliferation factor 2 (BTG2), 752–753  
 BTG2. *See* BTG antiproliferation factor 2 (BTG2)  
 Bullous pemphigoid (BP), 1218  
 Burosumab, 288–289
- C**  
 c-Jun N-terminal kinase (JNK), 979  
 c-MYC, 688–690  
 c-Myc oncogene, 688–690  
 c-MYCproto-oncogene, 754  
 C-reactive protein (CRP), 575, 1103  
 C-reactive protein, 218  
 C-ring analogs, 1118–1119  
 C-terminal telopeptide (CTx), 164, 414, 1271–1272  
 C/EBP  $\alpha$ . *See* CCAAT/enhancer binding protein  $\alpha$  (C/EBP  $\alpha$ )  
 C3-epimerization, 664  
 C3-epimers, 665  
 C3. *See* Complement 3 (C3)  
 C5a. *See* Complement factor 5a (C5a)  
 CA. *See* Carnosic acid (CA)  
 CaBP. *See* Calcium-binding protein (CaBP)  
 CAC. *See* Colitis-associated colorectal carcinogenesis (CAC)  
 Cadherin EGF LAG sevenpass G-type receptor 2 (CELSR2), 189  
 CADM2. *See* Cell adhesion molecule 2 (CADM2)  
 CAFs. *See* Cancer-associated fibroblast (CAFs)  
 Calbindin, 512  
   28K, 694–695  
 Calcidiol (25(OH)D), 480  
 Calcidiol treatment, 345–349  
 Calcifediol, 457, 1103  
   function of 25(OH)D<sub>3</sub>/1,25(OH)<sub>2</sub>D<sub>3</sub> axis, 458t  
   serial link amongcalcifediolCYP27B1, 465–466  
   treatment of microbial disease toboost human innate and adaptive immune responses, 465–469  
   concerted, serial link among calcifediolCYP27B1, calcitriol, vitamin D receptor, and human immuneresponse, 465–466  
   microbial infection and serum 25-hydroxyvitamin D status of host, 466–467  
   use of IRC and ERC in ARI including COVID-19, 467–469  
   treatment of secondary hyperparathyroidism associated with CKD, 462–465  
   pathophysiology of secondary hyperparathyroidism, 462  
   treatment of VDI in CKD, 462–465  
   treatment of vitamin D insufficiency with, 460–462  
 Calciferols, 341  
 Calcimimetic drugs, 587–588  
 Calcineurin inhibitors, 1119  
 Calciopenic group, 241–242  
 Calcipotriene, 1217  
 Calcipotriol, 639, 812, 870, 883–884, 956, 1214, 1223  
 Calcitonin, 263  
 Calcitriol, 11, 341, 359, 918, 958  
   serial link among, 465–466  
   short-term effects of treatment with, 332  
 Calcitriol-resistant rickets (CRR), 359–360  
 Calcium (Ca), 167–170, 223, 226, 253, 263–264, 303, 363–364, 383–384, 412, 416–423, 625–626, 652, 681, 995  
   absorption, 401–402  
   studies of, 385–387  
   calcium-deficient rickets, 262–263  
   calcium-phosphate homeostasis, 1231  
   deficiency, 167, 228, 245–246  
   homeostasis, 167, 664–665  
   intake, 242, 250  
   kidney stones, 619–620  
   levels, 361  
   primary fracture prevention with calcium and vitamin D, 422–423  
   stages of calcium deprivation leading to nutritional rickets and osteomalacia, 230f  
   stones, 619  
   formation, 619–620  
   supplementation, 265  
   systematic reviews and metaanalyses of supplemental vitamin D and calcium on incident fractures, 446  
   treatment and supplementation of, 264t  
   and vitamin D, 425–426  
   secondary fracture prevention with, 426  
 Calcium carbonate, 317  
 Calcium sensing receptor (CaSR), 874  
 Calcium-binding protein (CaBP), 569  
 Calcium-sensing receptor (CASR), 304, 459, 599–600  
 knockout mice, 225

- Calmette-Guérin bacillus (BCG), 632  
 CAMP. *See* Childhood Asthma Management Program (CAMP)  
 cAMP. *See* Cyclic adenosine monophosphate (cAMP)  
 CAMP gene, 466, 1000  
 Canada  
   vitamin D status in, 49  
   vitamin D supplement in, 120–123  
     percentage of Canadians, 122f  
     supplement use of Canadians, 121f  
 Canadian Community Health Survey (CCHS), 120  
 Canadian Food and Drug Regulations, 143–144  
 Canadian Health Measure Survey, 49  
 Canadian Multicentre Osteoporosis Study, 33  
 Canadian national nutrition survey, 120–122  
 Canadian supplement, 120  
 Cancer, 2, 17–19, 33, 36–37, 69, 389, 503, 797  
   angiogenesis and metastasis, 686–687  
   cells, 954  
   clinical studies, 683–686  
   epidemiology cancer risk of, 135  
   genetic variation and gene expression in  
     vitamin D pathway, 730–732  
   growth, development, and  
     carcinogenesis, 682–683, 684t–685t  
   incidence, 720–724  
     breast cancer, 721–722  
     colorectal cancer, 720–721  
     other cancers, 722–723  
     prostate cancer, 722  
     randomized controlled trial studies of  
       vitamin D and total cancer  
       incidence, 723–724  
   mortality, 720  
   mortality and survival, 724–730  
     circulating 25(OH)D, 725  
     randomized controlled trials,  
       728–730  
     studies of vitamin D intake, 728  
     studies using predicted 25(OH)D,  
       727–728  
     sun exposure, 724–725  
   parathyroid hormone–related peptide,  
     687–688  
   prognosis, 719  
   risk of, 214  
   vitamin D, 679–688  
     receptor, 679–682  
 “Cancer Punctuated Equilibrium” model,  
   860  
 Cancer stem cells (CSCs), 847, 880  
 Cancer-associated fibroblast (CAFs), 952,  
   956  
*Candida albicans*, 998, 1263  
 Candidate gene studies, 731  
 Candidiasis, 632  
 Canonical endocrine function of vitamin  
   D, 460  
*Cantherelles*, 84  
 Capping protein regulator and myosin 1  
   linker 1 (CARMIL1), 190  
 CAR-T cells. *See* Chimeric antigen  
   receptor-T cells (CAR-T cells)  
 Carbamazepine, 1261  
 Carbamoyl-phosphate synthase 1 (CPS1),  
   190  
 Carbohydrate response element–binding  
   protein (ChREBP), 560  
 Carcinogenesis, 692  
 Carcinomas, 825–826  
 CARD15, 1058  
 Cardiac failure, 248, 306  
 Cardiac function, 575–577  
 Cardiac transplant patients,  
   479–480  
 Cardiac transplantation, 479–480  
 Cardiac wheezing, 1233  
 Cardiomyocytes, 569–570  
 Cardiomyopathy, 248  
 Cardiopulmonary disease, 162  
 Cardiovascular (CV), 425–426  
   vitamin D effects on CV cells, 569  
 Cardiovascular calcification, 291  
 Cardiovascular disease (CVD), 2, 16, 69,  
   135, 218, 489, 568, 570–571, 605, 619,  
   669  
   events, 577  
   importance of early-life vitamin D  
     status for later obesity and  
     subsequent risks of, 501–503  
   mortality, 571  
   vitamin D metabolism and regulation  
     with special reference to,  
       568–569  
 Cardiovascular events  
   in large vitamin D supplementation  
     trials in general populations,  
       577–578  
   risk of, 214–215  
 Cardiovascular function, vitamin D effects  
   on parameters of, 575–577  
 Cardiovascular mortality, 573–574  
 Cardiovascular outcomes, genetic studies  
   on vitamin D and, 571–574  
 Cardiovascular risk factors, 607  
 Cardiovascular system, 20–21  
   adverse vitamin D effects on, 579–580  
   in HVDRR patients, 390  
 CARMIL1. *See* Capping protein regulator  
   and myosin 1 linker 1 (CARMIL1)  
 Carnosic acid (CA), 765, 770–772  
 Cartilage, 224  
 Cartilaginous plate, 252  
 Case–control studies, 40–41, 515–516,  
   526, 724–725, 830  
 Casein kinase (CK), 861  
 CaSR. *See* Calcium sensing receptor (CaSR)  
 CASR. *See* Calcium-sensing receptor  
   (CASR)  
 CASR gene, 310  
 Cat-scratch disease, 632  
 Catabolic enzyme, 1262  
 Catabolic pathway of 24-OHase,  
   inhibition of, 636–637  
 Catabolism in chronic kidney disease,  
   alterations in renal 1,25(OH)<sub>2</sub>D  
   synthesis and 25(OH)D and  
   1,25(OH)<sub>2</sub>D, 592–594  
 Catalase expression, 979  
 Cataract, 246  
 $\beta$ -catenin, 683, 861  
   paradox, 861–863  
 CatG. *See* Cathepsin G (CatG)  
 Cathelicidin, 1077, 1214, 1263–1264  
 Cathelicidin antimicrobial peptide  
   (CAMP), 460, 466, 995, 997, 1092  
 Cathepsin G (CatG), 1112  
 Cathepsin inhibitors, 687  
 Cats, 663–664, 670–671  
   role of vitamin D on non-skeletal health  
     in, 670–671  
 Causality, 31  
 Caveolin-1, 749–750  
 CBMCs. *See* Cord blood mononuclear cells  
   (CBMCs)  
 CC genotype, 1242  
 CCAAT/enhancer binding protein  $\alpha$  (C/  
   EBP  $\alpha$ ), 752  
 C–Cchemokine receptor type 7 (CCR-7),  
   1021  
 CCHS. *See* Canadian Community Health  
   Survey (CCHS)  
 CCR-7. *See* C–Cchemokine receptor type 7  
   (CCR-7)  
 CCR5. *See* Chemokine receptor 5 (CCR5)  
 CCTC-binding factor (CTCF), 753  
 CD. *See* Crohn’s disease (CD)  
 CD11b, 742  
*Cd14* gene, 746, 1000  
   monocyte marker, 742  
 CD248 gene, 191  
 CD4 T cells, 1019, 1048  
 CD4<sup>+</sup> type 1 regulatory T cells in MS and  
   vitamin D, interplay between,  
   1145–1149  
 CD4<sup>+</sup>FOXP3<sup>+</sup>T regulatory cells, 1137–1145  
   and dysfunction in MS, discovery of,  
   1137–1138  
 CD4<sup>+</sup>T lymphocyte, 1130  
 CD4<sup>+</sup>Tr1 cell  
   discovery and characteristics, 1146  
   interplay between 1,25(OH)<sub>2</sub>D<sub>3</sub>  
     and CD4<sup>+</sup>Tr1 cells in MS, 1149  
   and MS, 1146–1147  
 CD44, 562–563  
 CD4D T cell subsets, effects of vitamin D  
   on, 1038–1040  
   T cell plasticity, 1040  
   Tfh cells, 1040  
   Th1 cells, 1038  
   Th2 cells, 1038–1039  
   Th9 cells, 1039  
   Th17 cells, 1039–1040  
 CD4D Tr1 cell production, 1,25(OH)<sub>2</sub>D<sub>3</sub>  
   modulation of, 1147–1148  
 CD8 T cells, 1021, 1041  
 CD8<sup>+</sup> T cells, 1041  
 CDC20 genes, 923  
 CDC6/18, 757–758

- cDCs. *See* Conventional dendritic cells (cDCs)
- CDK inhibitory proteins (CDKIs), 756–757
- CDKIs. *See* CDK inhibitory proteins (CDKIs)
- CDKN1A* genes, 922
- CDKs. *See* Cyclin-dependent kinases (CDKs)
- CDMS. *See* Clinically definite MS (CDMS)
- CDT1, 757–758
- Cell adhesion molecule 2 (CADM2), 190
- Cell antigen receptor (BCR), 1036
- Cell cycle, 692–693
- checkpoint control, 692–693
  - events modulation by vitamin D and analogs, 758–763
  - cell type specificity, 763–764
  - downregulation of c-MYC expression in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced differentiation, 762–763
  - G1/S block, 759
  - G2/M retardation and polyploidization, 763
  - retinoblastoma protein control of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced G1 block, 762
  - upregulation of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, 759–762
- progression through G1 and G1/S boundary, 756–757
- VDDs effects on, 754–755
- autophagy and, 765
- Cell differentiation, 695, 874
- Cell metabolism, 846–847
- Cell proliferation, 871–873
- inhibition by vitamin D and analogs without evidence of differentiation, 763–764
- Cell survival, 873–874
- VDDs effects, 764–765
- Cell type
- specificity, 763–764
  - studies in, 372–373
- Cell-mediated adaptive immunity, 1035–1036
- Cells, 352
- Cellular adhesion, 692
- Cellular proliferation, 1262
- CELSR2* gene, 189
- CELSR2*. *See* Cadherin EGF LAG seven pass G-type receptor 2 (*CELSR2*)
- Centers for Disease Control and Prevention (CDC), 448–450, 511–512
- Central laboratory facility, multicenter and global studies using, 66
- Central nervous system (CNS), 171–172, 284, 1130, 1167–1168
- features, 638
- CETP* gene, 187–189
- CF. *See* Cystic fibrosis (CF)
- CFTR* mutation, 938
- CFU-GM–derived cells. *See* Granulocyte–macrophage colony-forming unit–derived cells (CFU-GM–derived cells)
- CHB. *See* Chronic hepatitis B (CHB)
- CHC. *See* Chronic hepatitis C (CHC)
- Checkpoints, 755
- Chemically induced vitamin D compounds efficacy to inhibiting chemically induced carcinogenesis, 839
- Chemically-induced colon/colorectal cancer models, 881–882
- Chemokine receptor 5 (CCR5), 1011–1012
- Chemokine responses, 1,25(OH)<sub>2</sub>D and antiviral cytokine and, 1018–1019
- Chemokines, 801
- Chemoprevention
- of NSCLC, 904–905
  - of PCa by vitamin D supplementation, 925–926
- Chemotherapy of PCa with 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs and impact of genomic ancestry, 926–927
- Chest radiographs, 232
- Chicken, 86
- Child abuse, 252
- Childhood asthma, 1236
- Childhood Asthma Management Program (CAMP), 1243–1244
- Childhood obesity, 502–503
- Childhood Origins of Asthma (COAST), 1242
- Childhood to adulthood, evolution of VDDR1A under treatment from, 332–335
- Childhood wheezing, 1233, 1236, 1242
- hypothetical prevalence of three different wheezing phenotypes in, 1234f
  - observational studies on ARI, including, 1236–1238
- Children, 236–237, 316, 361
- alopecia and rickets in children with HVDRR, 362f
  - with rickets, 248
- Chimeric antigen receptor-T cells (CAR-T cells), 692
- Chinese Longitudinal Health and Longevity Study (CLHLS), 19
- ChIP-seq. *See* Chromatin immunoprecipitation sequencing (ChIP-seq)
- Chloroquine, 631
- Cholangiocytes, 560–561
- Cholecalciferol, 42, 78–79, 125, 136, 341, 465, 609
- supplementation, 926, 1267
- Cholestatic liver disease, 249, 671
- Cholesterol, 20
- Chondrocyte-selective transcription factors, 225
- Chondrocytes, 224, 252
- Chorionic villus sampling (CVS), 385
- ChREBP. *See* Carbohydrate response element–binding protein (ChREBP)
- Chromatin immunoprecipitation sequencing (ChIP-seq), 745, 880, 1133
- Chromatographic methods, 48–49
- Chromosome 22q11.2 region, 306–309
- Chromosome 4q21, 291
- Chronic disease, 32–33, 116–117, 123, 161, 201, 1261
- Chronic granulomatous disease, 633
- Chronic hepatitis B (CHB), 799
- Chronic hepatitis C (CHC), 813
- Chronic hypocalcemia, 306
- Chronic hypophosphatemia, 284
- Chronic illness, 303–304
- Chronic immune-mediated disease, 1109
- Chronic inflammation, 797–798
- Chronic inflammatory enteropathy (CIE), 667–668
- Chronic kidney disease (CKD), 457, 476, 568–569, 578, 587, 589, 592–595, 602, 669
- alterations
    - 1,25(OH)<sub>2</sub>D production by extrarenal 1 $\alpha$ -Hydroxylases in chronic kidney disease, 594–596
  - calcifediol treatment of secondary hyperparathyroidism associated with, 462–465
- CKD-induced changes, 591
- 1,25(OH)<sub>2</sub>D corrects the impaired 25(OH)D availability to nonrenal 1 $\alpha$ -hydroxylase in, 596–599
- in 1,25(OH)<sub>2</sub>D/VDR endocrine and autocrine/paracrine actions in CKD, 596–599
- 1,25(OH)<sub>2</sub>D corrects impaired 25(OH)D availability to nonrenal 1 $\alpha$ -hydroxylase in CKD, 595f
- abnormal VDR regulation of gene transcription in CKD, 597f
- in renal 1,25(OH)<sub>2</sub>D synthesis and 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism in, 592–594
- pathogenesis of parathyroid hyperplasia and 1,25(OH)<sub>2</sub>D resistance in, 600f
- patients, 465
- progress to improve current recommendations for evaluation of vitamin D status and for design of safe and effective vitamin D interventions, 609–610
- role of 1,25(OH)<sub>2</sub>D deficiency in pathogenesis of secondary hyperparathyroidism in, 599f
- treatment of vitamin D insufficiency in, 462–465
- vitamin D
- control of hypertension and systemic inflammation conferring renal and cardiovascular protection independently of renal or systemic *klotho* levels, 606–609
  - control of secondary hyperparathyroidism, 599–602



- Chronic kidney disease (CKD) (*Continued*)  
 control of skeletal health in CKD  
   unrelated to attenuation of  
   secondary hyperparathyroidism,  
   602–603  
 control of skeletal health in CKD  
   unrelated to attenuation of  
   secondary hyperparathyroidism,  
   602–603  
 deficiency in patients with, 478  
 maintenance of renal FGF23/klotho  
   axis, 603–606
- Chronic liver diseases, 562, 813
- Chronic lymphocytic leukemia (CLL), 693
- Chronic obstructive pulmonary disease  
 (COPD), 1003–1004, 1245–1249  
   acute exacerbations of COPD,  
   1247–1248  
   AECOPD, 1249  
   pathogenesis and progression,  
   1245–1246, 1249  
   potential mechanisms, 1248
- Chronic obstructive pulmonary disease,  
 1234–1235
- Chronic pancreatitis (CP), 938
- Chronic rheumatic disorders, 797
- Chronic synovial inflammation, 1188
- Chronic myelogenous leukemia (CML),  
 751
- CI. *See* Confidence interval (CI)
- CIE. *See* Chronic inflammatory  
 enteropathy (CIE); International  
 Commission on Illumination (CIE)
- Cigarette smoke exposure, 906
- Cinacalcet, 384–385  
   therapy, 384–385
- Circulating 25(OH)D, 725  
   breast cancer, 726  
   colorectal cancer, 725–726  
   lung cancer, 727  
   other cancers, 727  
   prostate cancer, 726–727
- Circulating 25(OH)D, measures of, 33–34
- Circulating levels, 461t
- Circulating vitamin D nutritional status,  
   evolutionary perspective of, 15f
- Cirrhosis, 303–304, 638, 813
- CIS. *See* Clinically isolated syndrome (CIS)
- Cis-locked 1a, 25-dihydroxylumisterol3,  
 976–977
- Cis-regulatory elements, 1141, 1144
- Cis-trans relationships, 924
- Cis-urocanic acid, 972
- Cisplatin, 698–699
- Cistronic approaches, 923–925
- Citrate, 1011–1012
- Citrobacter rodentium*, 1065–1066
- Citrullinated histones (H3Cit), 1001
- CK. *See* Casein kinase (CK)
- CKD. *See* Chronic kidney disease (CKD)
- CKD-minera and bone disorder (MBD),  
 462
- Classic negative feedback loop, 412
- “Classical” genomic actions, 596–597
- Claudin-1, 1061
- CLDN16 gene, 312
- CLDN19 gene, 312
- CLHLS. *See* Chinese Longitudinal Health  
 and Longevity Study (CLHLS)
- Clinical applications of VDDs actions  
   against hematopoietic  
   malignancies, 768–774  
   clinical trials of VDDs in hematopoietic  
   malignancies, 772–774, 773t  
   differentiation therapy of leukemia, 768  
   doxercalciferol, 769, 774  
   paricalcitol, 774  
   potential mechanisms, 769–770  
   synthetic vitamin D analogs, 774  
   vitamin D analogs and functional  
   analysis, 768–769  
   vitamin D compounds in combination  
   with agents, 770–772
- Clinical disease, 667
- Clinically definite MS (CDMS), 1175–1177
- Clinically isolated syndrome (CIS),  
 1167–1168
- Clinical trials, 512  
   of TB, 1079  
   Wnt/ $\beta$ -catenin antagonism by  
   1,25(OH) $_2$ D $_3$  in animal models and,  
   870–871
- CLL. *See* Chronic lymphocytic leukemia  
 (CLL)
- CLMN gene, 692
- Clostridioides difficile*, 1068
- Clostridium*, 1060
- Cloud cover, 34
- CM. *See* Conditioned medium (CM)
- CML. *See* Chronic myelogenous leukemia  
 (CML)
- CMM. *See* Cutaneous malignant  
 melanoma (CMM)
- CMS. *See* Consensus molecular subtypes  
 (CMS)
- CMV. *See* Cytomegalovirus (CMV)
- CNS. *See* Central nervous system (CNS)
- Coactivator-binding, mutations affect,  
 379–380
- Coactivators, 652
- COAST. *See* Childhood Origins of Asthma  
 (COAST)
- Coccidioidomycosis, 632
- Cochrane Review, The, 682
- Cockayne syndrome, 971
- Cod liver oil, 77, 84
- Cognitive impairment, 1154
- Cohesin, 758
- Cohort studies, 39–40, 724–725
- Colitis-associated colorectal  
 carcinogenesis (CAC), 882
- Collagen I synthesis, 1217
- Colon cancer. *See also* Breast cancer (BCa)  
   effects of 1,25(OH) $_2$ D $_3$  in colon cancer-  
   associated fibroblasts, 878–880  
   effects of 1,25(OH) $_2$ D $_3$  in colorectal stem  
   cells—derived organoids, 880–881  
   effects on colon cancer cell proliferation,  
   survival, and differentiation,  
   871–874  
   genetics and subtypes, 859–861  
   human observational and intervention  
   studies, 863–864  
   mechanism of action of 1,25(OH) $_2$ D $_3$  in,  
   868–881, 868  
   vitamin D hydroxylases and VDR in,  
   864–868  
   Wnt/ $\beta$ -catenin signaling pathway,  
   861–863
- Colon cells, 863
- Colorectal cancer (CRC), 679–680,  
 720–721, 725–726, 802–803, 859  
 cells, 701–702
- Combination therapy, 697–699  
   glucocorticoids, 698  
   inhibitors of CYP24A1, 697  
   retinoids, 698
- Comel-Netherton syndrome, 1218
- Comet assays, 976
- Commensals, 1061–1062
- Complement 3 (C3), 996–997
- Complement factor 5a (C5a), 996–997
- Complement receptor immunoglobulin  
 (CRiG), 996–997
- Complementation group C protein (XPC),  
 971
- Complex genomic approaches, 593
- Compound heterozygous mutations in  
 VDR, 380
- Compromised respiratory function, 284
- Conditioned medium (CM), 877
- Confidence interval (CI), 415, 950
- Confounding  
   limitations of observational studies and  
   issue of, 526  
   limited to extent possible, 37–38
- Congenital disorders, 306
- Congenital rickets, 243
- Congenital vitamin D disorders, 666
- Congestive heart failure, vitamin D  
   deficiency in patients with, 477
- Conotruncal anomaly face syndrome,  
 306–309
- Consanguinity, 361, 375–376
- Consensus molecular subtypes (CMS),  
 860
- Conventional dendritic cells (cDCs), 1212
- Conventional therapy, 289–290, 317
- Convulsions, 329
- Cooking—antioxidant/pH, 89
- COPD. *See* Chronic obstructive pulmonary  
 disease (COPD)
- Cord blood leptin, 499
- Cord blood mononuclear cells (CBMCs),  
 1040
- Corepressors, 699–700
- Coronavirus, 1091
- Coronavirus-induced disease 2019  
 (COVID-19), 22–26, 217, 503, 1012,  
 1014–1015, 1091  
   community observational studies,  
   1095–1099  
   selected studies investigating vitamin  
   D deficiency and COVID-19,  
   1096t–1098t

- current guidance of COVID-19 and vitamin D and future research, 1104
- hospital observational studies, 1099
- infection, 23
- pandemic, 389, 596, 1232
- relevance to COVID-19 infection, 1091–1094
- use of IRC and ERC in ARI including, 467–469
- vaccinations, 1045
- vitamin D and, 1094
- protective effect of 1,25(OH)<sub>2</sub>D
- against sars-cov-2 infection, 1094f
- vitamin D supplementation and
- COVID-19, 1100–1104, 1101t–1102t
- Correction of vitamin D deficiency, 258
- Cortical microstructural properties, 165
- Corticosteroids, 1243
- resistance, 1244
- responsiveness to, 1243–1244
- COSMOS study, 601–602
- Costochondral junctions, 246
- Cotransfection of SRC-1, 379
- Country-level guidelines for supplements use, 118–120
- Covered transport, 105
- COVID-19. *See* Coronavirus-induced disease 2019 (COVID-19)
- Cows, 85
- COX. *See* Cyclooxygenase(COX)
- Cox regression analysis, 538–539
- Cox survival method, 1014–1015
- COX-2/PGE<sub>2</sub> pathway, 810
- CP. *See* Chronic pancreatitis (CP)
- CPDs. *See* Cyclobutane pyrimidine dimers (CPDs)
- CpG. *See* Cytosine–phosphate–guanine (CpG)
- CPS1. *See* Carbamoyl-phosphate synthase 1 (CPS1)
- Cramp* gene, 1000
- Craniosynostosis, 246–247
- Craniotabes, 246–247
- CRC. *See* Colorectal cancer (CRC)
- Creatinine clearance, 333
- CREB. *See* Cyclic AMP response–binding element protein (CREB)
- CRlg. *See* Complement receptor immunoglobulin (CRlg)
- CRISPR/Cas9 technology, 657–658
- Critical care, 670
- Critical illness
- severity of critical illness and organ failure, 1261
- vitamin D mechanisms in, 1262–1265
- Critically ill patient
- prevalence of vitamin D deficiency in, 1260
- vitamin D deficiency risk factors in, 1260–1261
- chronic disease, 1261
- ethnicity, 1260
- medication usage, 1261
- seasonality and sun exposure, 1260–1261
- severity of critical illness and organ failure, 1261
- Crohn's disease (CD), 295, 802–803, 859, 996, 998–999, 1057–1058
- Cross-sectional studies, 515–516, 526
- CRP. *See* C-reactive protein (CRP)
- CRR. *See* Calcitriol-resistant rickets (CRR)
- Crypts, 870–871
- Crystal form and dissolved in organic solvents, antirachitic compounds
- vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in, 78–79
- Crystals of vitamin D<sub>2</sub>, 79
- CSCs. *See* Cancer stem cells (CSCs)
- CST5 gene, 871–872
- CTA091, 909
- CTCF. *See* CCTC-binding factor (CTCF)
- CTLA-4. *See* Cytotoxic T lymphocyte antigen-4 (CTLA-4)
- Ctla4* gene, 1130–1131, 1138–1139
- CTLs. *See* Cytotoxic T lymphocytes (CTLs)
- CTRC mutation, 938
- CTX. *See* Cyclophosphamide (CTX)
- Cultured skin fibroblasts, initial studies using, 365–372
- Curcumin, 699, 771–772, 959
- Cutaneous malignant melanoma (CMM), 967
- Cutoffs, 2
- CVD. *See* Cardiovascular disease (CVD)
- CVS. *See* Chorionic villus sampling (CVS)
- Cyclic adenosine monophosphate (cAMP), 304
- Cyclic AMP response–binding element protein (CREB), 983
- Cyclin B, 758
- Cyclin D, 756
- Cyclin-dependent kinases (CDKs), 688–690, 755, 871
- Cyclins, 755
- Cyclobutane pyrimidine dimers (CPDs), 968–969
- Cyclooxygenase(COX), 805
- COX-1, 805
- COX-2, 799, 805
- Cyclophosphamide (CTX), 1114
- Cyclosporin, 1067
- Cyclosporine, 1119
- CYP. *See* Cytochrome P450 (CYP)
- CYP11A1, 972–973
- CYP27B1. *See* Cytochrome P450 family 27 subfamily B member 1 (CYP27B1)
- CYP2J2 gene, 186–187
- CYP2R1, 244, 342–349, 354, 413–414, 730
- associations between CYP2R1 single nucleotide polymorphisms and serum levels, 354t
- associations between CYP2R1 variants and disease, 353–354
- expression, 546
- gene, 43, 101–102, 186–187, 192–193, 201–202, 213–216, 327–328, 342, 349, 363, 385, 864–865, 1110–1111, 1132, 1172
- mutations, 361, 387
- biochemical and clinical features of subjects with, 346t–347t
- predicted variant effects, 350t–351t
- principal human vitamin D 25-hydroxylase, 342–345
- regulation of CYP2R1 in health and disease, 354–355
- in vitro and in silico analyses of CYP2R1 function, 352–353
- CYP3A4, 342, 349–352, 363
- oxidizes 25-hydroxyvitamin D, 352
- CYP3A4. *See* Cytochrome P450 3A4 (CYP3A4)
- Cystic fibrosis (CF), 476–477
- Cystinosis, 284–285
- Cytochrome P450 3A4 (CYP3A4), 1014, 1261
- Cytochrome P450 family 2 subfamily R member 1 (CYP2R1), 191, 1012–1013
- Cytochrome P450 family 24 subfamily A member 1 (CYP24A1), 192
- Cytochrome P450 family 27 subfamily B member 1 (CYP27B1), 304, 341, 457–458, 699–701, 802, 807, 837, 975, 995–996, 1015–1016, 1092, 1210–1211
- expression, 459
- gene, 282–283, 329–330, 360–361, 385, 412, 864–865, 995–996, 1000, 1077, 1110, 1113, 1131–1133, 1139, 1151, 1172–1174
- mutations, 387, 402
- Cytochrome P450 (CYP), 33, 186–187, 330, 402–403, 664, 798, 1151
- Cytochrome P450 family 24 subfamily A member 1 (CYP24A1), 192, 401, 571–572, 1012–1013
- clinical features, 404–405
- diagnosis, 405
- enzyme, 457–458
- expression, 958
- gene CYP24A1, 128, 201–202, 214, 216, 258, 288, 342, 401–403, 413–414, 457–458, 620–621, 634, 692, 699–700, 807, 837, 1077, 1110, 1131–1132, 1174, 1210–1211
- encoding 24-OHase enzyme, 637
- expression, 459
- polymorphisms in CYP24A1 gene and cardiovascular outcomes, 574
- genetic basis of disease, 402–403
- historical notes, 401–402
- 24-hydroxylase and vitamin D metabolism, 403f
- molecular genetics of, 402–403
- mutations, 401–402, 404–405
- pathophysiology of disease, 403–404
- promoter in osteoclast precursors, TAF12 binds, 655
- therapy, 405–407
- transcripts, 1137
- Cytokines, 634
- genes, 1059

- Cytokines (*Continued*)  
 1,25(OH)<sub>2</sub>D promotes expression of, 999–1000  
 Cytomegalovirus (CMV), 799–801, 1146–1147  
 Cytosine–cytosine dimers, 968–969  
 Cytosine–phosphate–guanine (CpG), 33  
 Cytostatic drug, 698–699  
 Cytostatic drugs, 770  
 Cytotoxic  
   CD8<sup>+</sup>T cells, 798–799  
   cells, 1186–1188  
   drugs, 770  
 Cytotoxic T lymphocyte antigen-4 (CTLA-4), 954  
 Cytotoxic T lymphocytes (CTLs), 767, 954, 1021
- D**  
 D deficiency  
   in children, 242  
   with HVDRR, 362f  
   development of, 306f  
   differential diagnosis of rickets from imaging perspective, 235–236  
   growth plate in, 225–226, 252  
   imaging choice in, 236–237  
   knee X-rays with typical signs of, 236f  
   of prematurity or metabolic bone disease of prematurity, 263–265  
   radiographic differences between forms of, 231–236  
   radiological features of nutritional rickets, 231–232  
   radiological features of osteomalacia, 232  
   radiological signs of nonnutritional rickets/osteomalacia, 232–235  
   resistant to vitamin D therapy, 327  
 1-β-d-arabinofuranosyl cytosine (AraC), 770  
 D-Health Trial, 42, 427–428, 539, 577–578, 723, 729  
 D-limonene, 959  
 D-ring analogs, 1118–1119  
 D-supplements, 118  
 D2d, 541  
   study, 542–544  
 DAG. *See* 1,2-diacylglycerol (DAG)  
 Daily dietary intake of vitamin D, 77  
 DAISY. *See* Diabetes Auto-Immunity Study in Young (DAISY)  
 Damage-associated molecular patterns (DAMP), 460, 995, 1012, 1185–1186  
 DAMP. *See* Damage-associated molecular patterns (DAMP)  
 Dark CPDs, 970  
 Dark skin pigmentation, 1260  
 DAS28. *See* Disease activity score 28 joints (DAS28)  
 DBD. *See* DNA-binding domain (DBD)  
 DBP. *See* Vitamin D–binding protein (DBP)  
 DBS. *See* Dried blood spots (DBS)  
 DC-STAMP domain containing 2 (DCST2), 189  
 DCIS. *See* Ductal carcinoma in situ (DCIS)  
 DCs. *See* Dendritic cells (DCs)  
 DCST2. *See* DC-STAMP domain containing 2 (DCST2)  
 De novo lipid synthesis (DNL), 560  
 De novo lipogenesis (DNL), 560  
 “Deep” subcutaneous adipose tissue, 490  
 Defb2 gene, 1000  
 Defective mineralization, 223  
 Deformities, 246  
 7-dehydrocholesterol reductase (DHCR7), 589  
 7-dehydrocholesterol (7DHC), 78, 101–102, 125, 136, 341, 413–414, 663–664, 1048–1049, 1076, 1208–1209, 1260  
 DEJ. *See* Dermal–epidermal junction (DEJ)  
 Delay in tooth eruption, 247  
 Deletions, 306–309  
 Deletions, 376–377  
 Demyelinating disease, 1130  
 Dendritic cells (DCs), 745, 1013–1014, 1016f, 1035–1036, 1044, 1062, 1092, 1113, 1185–1186  
 Dengue fever, 1013–1014  
 Denmark, 83  
 Denosumab, 482, 687  
 Densitometry (DXA), 228–229  
 Dental caries, 1002–1003  
 Dentin matrix protein I (DMP1), 604  
 Deoxyypyridinoline, 250  
 Depression, 217  
 DEQAS. *See* Vitamin D External Quality Assessment Scheme (DEQAS)  
 Dermal–epidermal junction (DEJ), 1219  
 Dermatitis herpetiformis (DH), 1218  
 Dermatology, 670  
 Desmoglein 1 (Dsg 1), 1218  
 Desmoglein 3, 1218  
 Detoxification, 877–878  
 Detoxifying effects, 874–878  
 Dexamethasone, 562–563, 698, 1095–1099  
 Dextran sulfate sodium (DSS), 807, 875, 996, 1064–1066  
 DGAT2. *See* Diacylglycerol O-acyltransferase 2 (DGAT2)  
 DH. *See* Dermatitis herpetiformis (DH)  
 DHCR7, 730  
   DHCR7-NADSYN1 gene, 216  
   gene, 43, 101–102, 186–187, 201–202, 213–215, 1110, 1132  
 DHCR7. *See* 7-dehydrocholesterol reductase (DHCR7)  
 DHT. *See* Dihydrotachysterol (DHT)  
 Diabetes, 162, 511–512, 1113–1114, 1119–1120  
   mellitus, 69, 511–512, 619  
   prevention trials, 543  
 Diabetes Auto-Immunity Study in Young (DAISY), 1110  
 Diabetes Prediction and Prevention (DIPP), 1110  
 Diabetes Prevention Program (DPP), 544  
   cohorts, 544  
 Diabetes prevention with active Vitamin D (DPVD), 511, 538–539, 541  
   study, 538–539  
 Diabetic kidney disease, 605  
 Diacylglycerol O-acyltransferase 2 (DGAT2), 191  
 1,2-diacylglycerol (DAG), 746–747  
 DIDS. *See* 4,4-diisothiocyantostilbene-2,2-disulfonic acid (DIDS)  
 Diet, 161  
   studies, 881–882  
 Dietary calcium, 304–305, 639–640  
 Dietary calcium deficiency, 258–263, 314  
   clinical presentation of children with, 260f  
 Dietary change, 1060  
 Dietary data, 37  
 Dietary habits, 622  
 Dietary intake, 282  
   of vitamin D and MS risk, 1169  
 Dietary phosphate, 284  
 Dietary phosphate restriction, 592  
 Dietary recommendations  
   to meet status gap, 136  
   for nutrient, 11  
 Dietary reference intakes (DRIs), 3, 127  
 Dietary strategies for increasing vitamin D intake, 143–148  
   dose-response data, 147f  
   hierarchy of evidence, 145–148  
   national case study, 144–145  
   traditional food fortification with vitamin D, 143–144  
 Dietary sugars, 560  
 Dietary supplementation, 959  
 Dietary supplements solution, 137–138  
 Dietary vitamin D, 664  
   effect, 840–841  
 Dietary modeling analysis, 148, 151–153  
 Differentially methylated region (DMR), 1134  
 Differentiation  
   activation of VDR as initial VDD-induced signal for, 742–746  
   cell proliferation inhibition by vitamin D and analogs without evidence of, 763–764  
   developmental, 752–755  
   of myeloid leukemia cells by VDDs, 741–742  
   and markers, 742  
   signaling pathways activated by VDDs in acute myeloid leukemia cells, 746  
   JAK/STAT pathway, 752  
   mitogen-activated protein kinase pathway, 746–747  
   phosphoinositide 3-kinase/AKT pathway, 747–749  
   protein kinase C signaling, 746–747  
   Wnt/β-catenin signaling pathway, 751  
   therapy of leukemia, 768  
 DiGeorge syndrome, 306–309  
 dihydro-TST. *See* Dihydrotestosterone (dihydro-TST)

- Dihydrotachysterol (DHT), 639  
 Dihydrotestosterone (dihydro-TST), 1139  
 1 $\alpha$ ,25-dihydroxyvitamin D [1,25(OH) $_2$ D], 359  
 24,25-dihydroxyvitamin D (24,25(OH) $_2$ D), 620, 1076  
 25-dihydroxyvitamin D $_2$ , 959  
 24,25-dihydroxyvitamin D $_3$  (24,25(OH) $_2$ D $_3$ ), 457  
 Dihydroxyvitamin D $_3$ , 976–977  
 4,4-diisothiocyanatostilbene-2,2-disulfonic acid (DIDS), 978  
 Dilated cardiomyopathy, 228, 248  
 1,2-dimethylhydrazine dihydrochloride (DMH), 882  
 DIPART. *See* Vitamin D Individual Patient Analysis of Randomized Trials (DIPART)  
 DIPP. *See* Diabetes Prediction and Prevention (DIPP)  
 Disease  
   microbiota in, 1059–1060  
   regulation of CYP2R1 in, 354–355  
 Disease activity score 28 joints (DAS28), 1196  
 Disease end point of interest, time period for assessment of vitamin D status covered based on pathogenesis of, 36–37  
 Disease-modifying antirheumatic drugs (DMARDs), 1200  
 Disorders, 295  
 Diuretics, 265, 575  
 Dizygotic twins (DZ), 186  
 DKK1, upregulation of, 869–870  
 DKK4, downregulation of, 869–870  
 DM. *See* Dry matter (DM)  
 DMARDs. *See* Disease-modifying antirheumatic drugs (DMARDs)  
 DMH. *See* 1,2-dimethylhydrazine dihydrochloride (DMH)  
 DMP1. *See* Dentin matrix protein I (DMP1)  
 DMP1 gene, 291  
 DMR. *See* Differentially methylated region (DMR)  
 DNA  
   direct DNA damage by absorption of UV, 968–969  
   DNA damage by UV exposure, 969f  
   DNA repair, 971–972  
     BER, 971–972  
     nucleotide excision repair, 971  
   DNA repair proteins and processes, 981–982  
   indirect DNA damage by UV, 969–970  
   licensing, 757–758, 757f  
   reduction in DNA damage and enhancement of repair, 976  
   repair, 692–693  
   UV-induced DNA damage, repair, and immune suppression, 968–972  
 DNA methylation, 1149–1155, 1153f  
 DNA methyltransferase genes 1 (Dnmt1), 1152  
 DNA-binding domain (DBD), 364, 373f  
   mutations in VDR DNA binding domain, 374  
 DNA-methyltransferase-3a (DNMT3a), 1130–1131, 1152  
 DNL. *See* De novo lipid synthesis (DNL); De novo lipogenesis (DNL)  
 Dnmt1. *See* DNA methyltransferase genes 1 (Dnmt1)  
 DNMT3a. *See* DNA-methyltransferase-3a (DNMT3a)  
 DO-Health, 577–578  
   study, 450  
   trial, 450–451  
 Does, 1239, 1268  
   frequency, 437  
 Dog, 663–664, 666–670  
   acquired vitamin D disorders, 666–667  
   congenital vitamin D disorders, 666  
   role of vitamin D on non-skeletal health in, 667–670  
 Dominant negative mutations, 380–381  
   “Dose–effect” relationship, 402–403  
 Dose–response metaanalysis, 727  
 Dose–response relationship, studies ideally assess, 37  
 Double blinded trial, 41  
 Downregulation  
   of c-MYC expression in 1,25(OH) $_2$ D $_3$ -induced differentiation, 762–763  
   of DKK4, 869–870  
 Dox. *See* Doxercalciferol (Dox)  
 Doxercalciferol (Dox), 630, 765, 769, 774  
   PRI-1906 and derivatives, 769  
 DPP. *See* Diabetes Prevention Program (DPP)  
 DPVD. *See* Diabetes prevention with active Vitamin D (DPVD)  
 DRB1\*1501 risk gene, 1134  
 Dried blood spots (DBS), 1171  
 DRIP. *See* Vitamin D receptor interacting protein (DRIP)  
 DRIs. *See* Dietary reference intakes (DRIs)  
 Drugs, 11  
   drug-resistant strains, 1075  
   nutrition clinical trials cannot match quality of evidence achieved with, 15–17  
 Dry matter (DM), 82  
 Dsg 1. *See* Desmoglein 1 (Dsg 1)  
 DSS. *See* Dextran sulfate sodium (DSS)  
 Dual-energy X-ray absorptiometry (DXA), 164, 229, 447  
 Ductal carcinoma, 825–826  
 Ductal carcinoma in situ (DCIS), 810, 825–826, 831  
 Duodenal switch, biliopancreatic diversion with, 165  
 Durvalumab, 903  
 Dutch National Institute for Public Health and Environment, 100  
 Dutch study, 451  
 DXA. *See* Densitometry (DXA); Dual-energy X-ray absorptiometry (DXA)  
 Dysbiosis, 876  
 Dyslipidemia, 501  
 Dyslipoproteinemia, 574–575  
 DZ. *See* Dizygotic twins (DZ)
- ## E
- E cadherin, 1061  
   induction of, 868–869  
 E2 response elements (EREs), 1143–1144  
 E47, 867  
 EA. *See* European American (EA)  
 EAAT3, 846  
 EAE. *See* Experimental autoimmune encephalomyelitis (EAE)  
 EAR. *See* Estimated average requirement (EAR)  
 Early neonatal hypocalcemia, 312  
 Early-infancy phenotype, 404  
 Early-onset CRC (EOCRC), 859  
 Early-stage lung cancer, vitamin D in management of, 905  
 EB1089 (vitamin D analog), 687, 694–695, 764, 958  
 EBA. *See* Epidermolysis bullosa acquisita (EBA)  
 EBM. *See* Evidence-based medicine (EBM)  
 EBV. *See* Epstein–Barr virus (EBV)  
 ECE-1. *See* Endothelin-converting enzyme 1 (ECE-1)  
 ECM. *See* Extracellular matrix (ECM)  
 Ecologic studies, 38–39, 720  
 Ecto-nucleotide pyrophosphatase/pyrophosphodiesterase 1 (ENPP1), 291  
 Ectopic soft-tissue calcification, 639  
 ECTS. *See* European Calcified Tissue Society (ECTS)  
 Eczema, 1240–1241  
 EDSS. *See* Expanded Disability Status Scale (EDSS)  
 EFSA. *See* European Food Safety Authority (EFSA); European Food Standards Agency (EFSA)  
 EFTUD1 gene, 692  
 EGF receptor (EGFR), 600–601  
 EGFR. *See* EGF receptor (EGFR); Epidermal growth factor receptor (EGFR)  
 eGFR. *See* Estimated glomerular filtration rate (eGFR)  
 EGFR mutations, 906  
 EGFR TKIs. *See* EGFR tyrosine kinase inhibitors (EGFR TKIs)  
 EGFR tyrosine kinase inhibitors (EGFR TKIs), 907  
 EGFR-LP. *See* EGFR-targeted lipid based nanoparticle (EGFR-LP)  
 EGFR-targeted lipid based nanoparticle (EGFR-LP), 910  
 Eggs, vitamin D in food farmed by, 84–87  
 EHT. *See* Endothelial-to-hematopoietic transition (EHT)  
 Eicosapentaenoic acid, 1200–1201  
 EKG. *See* Electrocardiogram (EKG)  
 Eldecacitol, 427  
 Electrocardiogram (EKG), 306, 638



- Electrophoretic mobility shift assays (EMSA), 378
- “Elfin-like” facies, 402
- ELISA. *See* Enzyme linked immunosorbent assay (ELISA)
- ELN gene, 402
- Empty vector (EV), 654–655
- Empty vector-transfected cells (EV–NIH3T3), 656
- EMSA. *See* Electrophoretic mobility shift assays (EMSA)
- EMT. *See* Epithelial-to-mesenchymal transition (EMT)
- End of treatment, 464–465
- End-stage heart failure, 568–569, 578
- Vitamin D in, 578
- End-stage pulmonary disease, vitamin D deficiency in patients with, 476–477
- End-stage renal disease, 599
- Endochondral bone, 224
- Endocrine, 1260
- diseases, 669
- society, 97, 446–447, 462
- vitamin D
- operating system, 459
- pathophysiology of endocrine vitamin D insufficiency, 460
- Endocytosis process, 591
- Endogenous
- characteristics, 101–102
- effect of age, 101
- genetics, 101–102
- obesity, 101
- dysregulation of vitamin D metabolites, 630
- overproduction, 667
- Endogenous vitamin D toxicity, forms of, 630–634
- endogenous production of 1,25-dihydroxyvitamin D, 630–634
- foreign material, 632
- granulomatous diseases, 630–631
- granulomatous diseases, 632–633
- lymphoma, 633–634
- sarcoidosis, 631
- tuberculosis, 632
- endogenous production of 25-hydroxyvitamin D, 630
- mechanisms of vitamin D toxicity, 634–638
- control of renal 1 $\alpha$ -OHase in vitamin D toxicity, 636
- general mechanisms, 634–635
- inhibition of catabolic pathway of 24-OHase, 636–637
- role of vitamin D receptor in vitamin D toxicity, 635–636
- vitamin D–binding protein and level of free metabolite in vitamin D toxicity, 637–638
- Endoplasmic reticulum (ER), 560–562
- ER stress–induced hepatic very-low-density lipoprotein receptor, 560
- Endothelial cells, 569
- Endothelial-to-hematopoietic transition (EHT), 752
- Endothelin-converting enzyme 1 (ECE-1), 286–287
- Energy, 982
- metabolism, 693–695
- Enhanced RANKL production in response to 1,25(OH) $_2$ D $_3$ , marrow stromal cells derived from P62<sup>P394L</sup>-knock-in mice and P62<sup>P392L</sup> Paget’s patients hyperresponsive to 1,25(OH) $_2$ D $_3$  and display, 657
- ENPP1 gene, 291
- ENPP1. *See* Ecto-nucleotide pyrophosphatase/pyrophosphodiesterase 1 (ENPP1)
- ENS. *See* Epidermal nevus syndrome (ENS)
- Enthesopathy, 288
- Envelope (E), 1091
- Enzyme linked immunosorbent assay (ELISA), 665–666
- Enzymes, 1036
- EOCRC. *See* Early-onset CRC (EOCRC)
- EpCAM. *See* Epithelial CAM (EpCAM)
- EPI. *See* Exocrine pancreatic insufficiency (EPI)
- 3-epi-1,25(OH) $_2$ D $_3$ , 664–665
- EPIC. *See* European Prospective Investigation into Cancer and Nutrition study (EPIC)
- Epidemiologic study designs, 31
- Epidemiological studies, 560
- association between vitamin D insufficiency and viral disease in, 1012–1015
- Epidermal growth factor receptor (EGFR), 872–873, 902–903
- Epidermal nevus syndrome (ENS), 285t, 292
- Epidermal stem cells, 741
- Epidermis, 101
- Epidermolysis bullosa acquisita (EBA), 1218
- Epigallocatechin gallate, 959
- Epigenetic(s)
- determinants, 33, 38
- regulation of gene expression, 1134
- of vitamin D, 1264–1265
- Epilepsy, 669
- Epithelial CAM (EpCAM), 953
- Epithelial-to-mesenchymal transition (EMT), 804, 860, 907, 953
- Epstein–Barr virus (EBV), 372–373, 700, 1013, 1167–1168
- EQAs. *See* External Quality Assessments (EQAs)
- ER. *See* Endoplasmic reticulum (ER); Estrogen receptor (ER)
- ER alpha. *See* Estrogen receptor alpha (ER alpha)
- ERC. *See* Extended release calcifediol (ERC)
- EREs. *See* E2 response elements (EREs)
- Ergocalciferol, 78–79, 136, 341, 417–418, 465, 609
- Ergosterol, 78
- ERK. *See* Extracellular–signal regulated kinase (ERK)
- ERK5, 750–751
- Erlotinib, 907
- ERp57, 976
- Erythroblastosis, 248
- Erythrocyte sedimentation rate (ESR), 1200
- Erythroleukemia cell line K562, 694
- Escherichia coli*, 997–998, 1263
- ESR. *See* Erythrocyte sedimentation rate (ESR)
- Estimated average requirement (EAR), 136, 242–243
- Estimated glomerular filtration rate (eGFR), 468–469
- Estradiol, 697–698, 1131
- Estrogen receptor (ER), 386–387, 728
- Estrogen receptor alpha (ER alpha), 691, 810, 825–826, 1135
- Estrogen(s), 172, 387
- support protective pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cell differentiation, 1139–1140
- Estrogenic hormones, 1139
- Ethnicity, 66, 948, 1047, 1260
- inflection points for PTH in relation to 25(OH)D by, 6f
- ETS fusion genes, 918
- ETS variant transcription factor 2 (ETV2), 752
- ETV2. *See* ETS variant transcription factor 2 (ETV2)
- ETV7 transcription factors, 754
- Eucalcemia, vitamin D in long-term maintenance of, 304–305
- Europe
- current intakes of vitamin D in young and adult populations in, 138–143
- vitamin D status in, 55, 56t–57t
- European American (EA), 918
- European Calcified Tissue Society (ECTS), 175–176
- European Food Safety Authority (EFSA), 128
- European Food Standards Agency (EFSA), 1, 5
- European pet food industry, The, 666
- European Prospective Investigation into Cancer and Nutrition study (EPIC), 720
- European Society of Pediatric Nephrology, 609
- EV. *See* Empty vector (EV)
- Everolimus, 749
- Evidence-based advice, 12–13
- Evidence-based medicine (EBM), 13
- EV–NIH3T3. *See* Empty vector-transfected cells (EV–NIH3T3)
- Evolutionary biology, 14–15
- Evolutionary perspective of circulating vitamin D nutritional status, 15f
- EWL. *See* Excess weight loss (EWL)

- Excess weight loss (EWL), 161–162  
 Exocrine pancreatic insufficiency (EPI), 668  
 Exogenous toxicity, 639  
 Exogenous vitamin D toxicity, forms of, 150, 625–630  
   1,25-Dihydroxyvitamin D toxicity, 628–629  
   toxicity due to synthetic vitamin D analogs, 629–630  
   vitamin D and 25(OH)D toxicity, 625–628  
 Expanded Disability Status Scale (EDSS), 1175, 1175t, 1177  
 Experimental autoimmune encephalomyelitis (EAE), 1133–1134, 1168–1169  
 Experimental studies, 41  
 Experimental uremia, 569  
 Extended release calcifediol (ERC), 457, 462, 467, 610  
   use of ERC in ARI including COVID-19, 467–469  
 External Quality Assessments (EQAs), 666  
 Extra-skeletal actions of vitamin D in HVDRR patients, 388–390  
   asthma in HVDRR patients, 389–390  
   autoimmune disease and cancer, 389  
   cardiovascular system in HVDRR patients, 390  
   extra-skeletal aspects of HVDRR, 388–389  
   immune system in HVDRR patients, 389  
   reproductive history of HVDRR patients, 390  
 Extracellular matrix (ECM), 848, 860, 952, 1212  
   protein 1, 1058  
 Extracellular phosphate, 281  
 Extracellular–signal regulated kinase (ERK), 981–982  
 Extrarenal 1 $\alpha$ -Hydroxylases in chronic kidney disease, alterations in 1,25(OH)<sub>2</sub>D production by, 594–596  
 Extravascular lung water index (EVLWI), 1269
- F**  
 F1P. *See* Fructose-1-phosphate (F1P)  
 FA. *See* Fatty acid (FA)  
 Fale calcitriol, 630  
 Falls, 435–438  
 FAM20C gene, 291  
 Familial adenomatous polyposis (FAP), 860  
 Familial benign chronic pemphigus. *See* Hailey–Hailey disease  
 Family-based studies, 186  
 Fanconi's syndrome, 284–285  
 FAO. *See* Food and Agriculture Organization (FAO)  
 FAP. *See* Familial adenomatous polyposis (FAP)  
 Far upstream element-binding protein 1 (FBP1), 761  
 Fat, 681  
   soluble vitamins, 668  
 Fat-soluble factor, 1079–1081  
 Fat-soluble factor A, 1079–1081  
 Fat-soluble vitamins, 11–12  
 FATP2. *See* Fatty acid transport protein 2 (FATP2)  
 Fatty acid (FA), 560  
 Fatty acid transport protein 2 (FATP2), 560  
 FBP1. *See* Far upstream element-binding protein 1 (FBP1)  
 FCA. *See* Fractional calcium absorption (FCA)  
 FD. *See* Fibrous dysplasia (FD)  
 FDA. *See* US Food and Drug Administration (FDA)  
 Fecal microbial transplantation (FMT), 1068  
 FEDIAF nutritional guidelines, 670  
 Feline VDDR-1A, 670  
 Female index, 345  
 Female–male differences provide insight into MS etiology, 1134–1135  
 Femoral neck (FN), 480–481  
 FEV1. *See* Forced expiratory volume in 1 (FEV1)  
 FFAs. *See* Free fatty acids (FFAs)  
 FGF23. *See* Fibroblast growth factor 23 (FGF23)  
 FGF7. *See* Fibroblast growth factor 7 (FGF7)  
 Fibroblast growth factor 23 (FGF23), 233, 282, 361, 459, 568, 574–575, 587–588, 592–593, 664, 1067  
   acquired hypophosphatemic disorders with elevated FGF23 levels, 293  
   genetic hypophosphatemic disorders with low FGF23 levels, 292–293  
   genetic hypophosphatemic syndromes of excess FGF23, 285–292  
   mutations, 402  
 Fibroblast growth factor 7 (FGF7), 293  
 Fibroblast-like synoviocytes (FLSs), 1189  
 Fibroblasts, 1189  
 Fibrogenic markers, 562–563  
 Fibronectin, 282–283  
 Fibrosis, 813  
 Fibrous dysplasia (FD), 291–292  
 FIND. *See* Finnish Vitamin D Trial (FIND)  
 Finland, 144–145  
 Finnish epidemiological study, 681  
 Finnish health, 144–145  
 Finnish Maternity Cohort (FMC), 723, 1170–1171  
 Finnish Vitamin D Trial (FIND), 539, 577–578, 729  
 Fish liver oils, 125  
 Fitzpatrick's type I or II, 967–968  
 FLG antisense RNA 1 (FLG-AS1), 189  
 FLG-AS1. *See* FLG antisense RNA 1 (FLG-AS1)  
 Flow cytometry, 475–476  
 FLSs. *See* Fibroblast-like synoviocytes (FLSs)  
 5-fluorouracil (5-FU), 691, 871  
 FMC. *See* Finnish Maternity Cohort (FMC)  
 FMT. *See* Fecal microbial transplantation (FMT)  
 FN. *See* Femoral neck (FN)  
 Fn. *See* *Fusobacterium nucleatum* (Fn)  
 Fok1, 974, 1083  
   VDR polymorphism, 1084  
 Folic acid, 15  
 Follicular helper cells (Tfh cells), 1040  
 Follow-up and more endpoints, 726–727  
 Food  
   insecurity, 123  
   processing, 78, 87  
   processing summary, 89  
 Food, vitamin D in, 82–91  
   and calculation of total vitamin D activity, 89–91  
   farmed by man–fish, eggs, milk, meat mushrooms, and plants, 84–87  
   beef, 86  
   bread and alternative vitamin D products, 86–87  
   chicken, 86  
   mushrooms, 86  
   organs, 86  
   pork, 85–86  
   food-based strategies for vitamin D, 155  
   fortified food, 87  
   including LOQ, precision, and accuracy, principle for extraction, detection, and quantification of vitamin D in, 81–82  
   processing of food—effects on degradation of vitamin D, 87–89  
   cooking—antioxidant/pH, 89  
   effect of exposure to light—not UVB, 88  
   food processing summary, 89  
   processing treatments, 88–89  
   relative humidity, 88  
   storage oxygenation, 88  
   effect of temperature, 88  
   from wild fish and animal, 82–84, 83f  
 Food allergy, 1248  
 Food and Agriculture Organization (FAO), 99, 143  
 Food composition data, 86  
 Food databases, 91  
 Food fortification approaches, 148, 256–258  
   bridging gap by, 143–148  
   consideration of vitamin D—fortified foods, 148–150  
   current intakes of vitamin D in young and adult populations in North America and Europe and compare against dietary targets, 138–143, 139t–140t  
   dietary recommendations to meet status gap, 136  
   dietary Reference Values/Intakes for vitamin D, 137t  
   dietary strategies for increasing vitamin D intake, 143–148, 151t–153t

- Food fortification approaches (*Continued*)  
 dietary supplements solution, 137–138  
 Food-based strategies are required, 138  
 prevalence of low vitamin D status, 135–136  
 safety considerations and prerequisite data required prior to initiating public health measures, 154–155  
 sources of vitamin D and role of food in meeting nutritional requirements, 136–137  
 vitamin D–biofortified foods, 150–154  
 Forced expiratory volume in 1 (FEV1), 1003–1004, 1240  
 Forkhead box O6 (FOXO6), 189  
 Fortified food, 87  
 Fortifying foods, 146  
 FOXO6. *See* Forkhead box O6 (FOXO6)  
*Foxp* gene, 1135  
*FOXP3*  
 gene, 1130–1131, 1135, 1137, 1139  
 hormonal regulation of, 1137–1145  
 hormonal regulation of, 1140–1145  
 Fractional calcium absorption (FCA), 168, 383, 385–386, 386f  
 Fracture(s), 435–436, 438–440, 475  
 effects of active vitamin D analogs on, 427  
 large randomized controlled trials of supplemental vitamin D on, 444t–445t  
 metaanalysis of randomized clinical trials of supplementation with vitamin D plus calcium, 439f  
 metaanalysis of randomized clinical trials of supplementation with vitamin D, 440f  
 risk, 438–440  
 RCTs of effects of vitamin D supplementation on, 448–451  
 Vitamin D and, 438–440  
 Free fatty acids (FFAs), 560  
 “Free” vitamin D concentrations, 492–493  
 Fresenius Medical Care’s European database (EuCliD5), 462–463  
 Frizzled proteins (FZD), 751  
 Fructose-1-phosphate (F1P), 560  
*Fusobacterium nucleatum* (Fn), 876–877
- G**  
 G-actin polymerizes, 1262  
 G0S2, 872  
 G1, progression through, 756–757  
 G1/S block, 759  
 G2 and M phase transition, 758  
 G2/M retardation and polyploidization, 763  
 GAD. *See* Glutamic acid decarboxylase (GAD)  
 GADD45A gene. *See* Growth arrest and DNA damage45 gene (GADD45A gene)  
 GADD45G genes, 923  
 GAL4. *See* Galactose4 (GAL4)  
 Galactose4 (GAL4), 352  
 Galactosemia, 284–285  
 GALNT2. *See* N-acetyl galactosaminyltransferase 2 (GALNT2)  
 GALNT3 mutations, 294  
 Garlic extract, 959  
 Gas chromatographic method, 80  
 Gastric banding, 163  
 Gastric bypass, 162  
 Gastric inhibitory peptide (GIP), 174  
 Gastrointestinal (GI)  
 cancers, 937  
 diseases, 667–668  
 disorders, 313  
 immune system, 1062  
 malabsorption, 295  
 Gastrointestinal tract, 172–173, 1059, 1061  
 vitamin D maintains homeostasis of, 1063f  
 Gastrointestinal tract, 1076  
 GC gene, 101–102, 186–187, 201–202, 214, 216, 413–414  
 GC rs2282679 polymorphism, 731  
 Gc1F. *See* Group-specific component 1 fast (Gc1F)  
 GCMB. *See* Glial cells missing B (GCMB)  
 Gefitinib, 907  
 Geminin, 757–758  
 GEMM. *See* Genetically engineered mouse models (GEMM)  
 Gene  
 ontology analysis, 806  
 polymorphisms, 571  
 Gene expression, 732, 1265  
 regulation of gene expression by  
 1,25(OH)<sub>2</sub>D, 364–365  
 in vitamin D pathway, 730–732  
 common variation, 730–732  
 Gene set enrichment analysis (GSEA), 880  
 Gene–environment interaction (GxE), 192–193  
 Genetic association studies, 682  
 Genetic confounding, 38  
 Genetic determinants, 32–33  
 Genetic hypercalciuric stone-forming rat (GHS), 621  
 Genetic hyperphosphatemic disorders, 293–294  
 tumoral calcinosis, 293–294  
 Genetic hypophosphatemic disorders with low FGF23 levels, 292–293  
 NPT2a mutations, 292  
 NPT2c mutations, 292–293  
 Genetic hypophosphatemic syndromes of excess FGF23, 285–292  
 ARHR, 290–291  
 autosomal dominant  
 hypophosphatemic rickets, 290  
 FD, 291–292  
 hypophosphatemic rickets–X-linked hypophosphatemia, 285–290  
 LNSS and ENS, 292  
 Genetic instrument, 193  
 Genetic linkage analysis, 802  
 Genetic polymorphisms, 628, 1059  
 in vitamin D system, 682  
 Genetic risk score (GRS), 194, 414  
 Genetic studies, 571  
 on vitamin D and cardiovascular outcomes, 571–574  
 metabolic pathway of vitamin D with selected enzymes and encoding genes, 573f  
 MR studies, 571–574  
 polymorphisms in CYP24A1 gene and cardiovascular outcomes, 574  
 polymorphisms in VDR gene and cardiovascular outcomes, 574  
 Genetic susceptibility of PC, 938  
 Genetic variants, 185–186  
 Genetic variation  
 measure of genetic variation of circulating 25(OH)D, 36  
 in vitamin D pathway, 730–732  
 common variation, 730–732  
 gene expression, 732  
 in vitamin D system and RA, 1199–1200  
 Genetically engineered mouse models (GEMM), 921–922  
 Genetics, 101–102, 949, 1109  
 Genistein, 959  
 Genome Aggregation Database (gnomAD), 403  
 Genome-wide association studies (GWAS), 102, 185, 202, 344–345, 428, 589, 682, 802, 924, 938, 1131–1132, 1171–1174  
 of 25(OH)D, 186–187  
 Genomic profiling of VDR agonists in breast cancer model systems, 841–844  
 1,25(OH)<sub>2</sub>D<sub>3</sub> regulated genes, 841–842  
 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated gene expression, 843  
 in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated breast cancer cell lines, 842–843  
 Genotype, 1238  
 Geographic studies, 515  
 GFI1. *See* Growth factor independent 1 (GFI1)  
 GFI1B, 752  
 GFR. *See* Glomerular filtration rate (GFR)  
 Ghrelin, 173–174  
 GHS. *See* Genetic hypercalciuric stone-forming rat (GHS)  
 GHS-R. *See* Growth hormone secretagogue receptor (GHS-R)  
 GIP. *See* Gastric inhibitory peptide (GIP)  
 Glial cells missing B (GCMB), 309  
 Gln259Pro. *See* Glutamine to proline at amino acid 259 (Gln259Pro)  
 Global Cancer (GLOBOCAN), 939–947  
 Global Initiative for Chronic Obstructive Lung Disease (GOLD), 1234  
 Glomerular filtration rate (GFR), 459, 591  
 GLP-1. *See* Glucagon-like peptide-1 (GLP-1)  
 Glu420Lys. *See* Glutamic acid to lysine at amino acid 420 (Glu420Lys)  
 Glucagon-like peptide-1 (GLP-1), 172–174  
 Glucocorticoids, 640–641, 698, 1067, 1235

- Glucose, 560  
 homeostasis, 1261  
 intolerance, 512  
 transporter activity, 513–514
- Glucose-dependent insulinotropic polypeptide, 174–175
- Glucose-stimulated secretion of insulin (GSSI), 499–500
- Glutamate ionotropic receptor delta-type subunit 1 (GRID1), 191
- Glutamic acid decarboxylase (GAD), 1120
- Glutamic acid to lysine at amino acid 420 (Glu420Lys), 381
- Glutamine to proline at amino acid 259 (Gln259Pro), 378–379
- Glutathione (GSH), 1152
- Glutathione peroxidase, 979
- Glutathione s-transferase (GST), 653
- Gly33Asp mutation, 373–374
- Glycemia, 499–500
- Glycerol-3-phosphate, 283–284
- Glycogen synthase kinase-3 (GSK3), 771, 982
- Glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), 763
- Glycogen synthase kinase3- $\alpha/\beta$  (GSK3- $\alpha/\beta$ ), 861
- GM-CSF. *See* Granulocyte-macrophage colony-stimulating factor (GM-CSF)
- GNAS gene, 311
- gnomAD. *See* Genome Aggregation Database (gnomAD)
- GOLD. *See* Global Initiative for Chronic Obstructive Lung Disease (GOLD)
- Gonadal steroids, 172
- Graft rejection, effects of vitamin D on, 475–476
- Granulocyte-macrophage colony-stimulating factor (GM-CSF), 698, 1018  
 transcripts, 1134
- Granulocyte-macrophagecolony-forming unit-derived cells (CFU-GM-derived cells), 652–653
- Granulomatous  
 diseases, 630–633, 639  
 disorders, 628  
 process, 632
- Grassroots Health, 19
- Graves' disease, 310
- GRID1. *See* Glutamate ionotropic receptor delta-type subunit 1 (GRID1)
- Group-specific component (Gc). *See* Vitamin D binding protein (DBP)
- Group-specific component 1 fast (Gc1F), 1083
- Growth arrest and DNA damage45 $\alpha$ gene (GADD45A gene), 872
- Growth factor independent 1(GFI1), 752
- Growth factor receptors, 695–697
- Growth factors, 695–697
- Growth hormone, 171
- Growth hormone secretagogue receptor (GHS-R), 173–174
- Growth plate, 224  
 changes of severe rickets on growth plate histology, 226f  
 growth plate in rickets, 225–226  
 in rickets, 225–226, 252
- Growth retardation, 328
- Growth velocity, 250
- GRS. *See* Genetic risk score (GRS)
- GSEA. *See* Gene set enrichment analysis (GSEA)
- GSH. *See* Glutathione (GSH)
- GSK3- $\alpha/\beta$ . *See* Glycogen synthase kinase3- $\alpha/\beta$  (GSK3- $\alpha/\beta$ )
- GSK3. *See* Glycogen synthase kinase-3 (GSK3)
- GSK3 $\beta$ . *See* Glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ )
- GSSI. *See* Glucose-stimulated secretion of insulin (GSSI)
- GST. *See* Glutathione s-transferase (GST)
- Guanine (G), 969
- Guanylate cyclase 2E, pseudogene (GUCY2EP), 191
- GUCY2EP. *See* Guanylate cyclase 2E, pseudogene (GUCY2EP)
- Gut epithelial VDR, 875
- Gut epithelium, 1061–1062
- Gut microbiome, 938
- Gut-derived hormones, 172–175  
 ghrelin, 173–174  
 GLP-1, 174  
 glucose-dependent insulinotropic polypeptide, 174–175  
 peptide YY, 173
- GWAS. *See* Genome-wide association studies (GWAS)
- GxE. *See* Gene-environment interaction (GxE)
- ## H
- H2.0-like homeobox transcription factor (HLX), 754
- H2Aa, 1112
- H3Cit. *See* Citrullinated histones (H3Cit)
- HA. *See* Hyaluronan (HA); Hyaluronic acid (HA)
- HAD-4. *See* Human alpha defensin 4 (HAD-4)
- Hailey-Hailey disease, 1219–1220
- Hair diseases, 1220–1221
- Hairless gene (HR gene), 364, 388
- HAL. *See* Histidine ammonia-lyase (HAL)
- Hallmarks of cancer, 797
- HAMP. *See* Hepcidin antibacterial protein (HAMP)
- Harm, 11
- Harvard-based VITAL trial, 831
- HATs. *See* Histone acetyltransferases (HATs)
- Hazard ratio (HR), 414, 918
- HBD2 gene, 1000
- HBD2. *See* Human beta defensin 2 (HBD2)
- HBEC. *See* Human bronchial epithelial cell (HBEC)
- hCAP-18. *See* Human cationic antimicrobial protein (hCAP-18)
- hCAP18 gene, 1077
- HCC. *See* Hepatocellular carcinoma (HCC)
- HCIN1. *See* Hypercalcemia-1 (HCIN1)
- HCIN2. *See* Hypercalcemia-2 (HCIN2)
- HCMV. *See* Human cytomegalovirus (HCMV)
- HCV. *See* Hepatitis C virus (HCV)
- HCY. *See* Homocysteine (HCY)
- HDAC. *See* Histone deacetylase (HDAC)
- HDPs. *See* Host defense peptides (HDPs)
- Head and neck squamous cell carcinoma (HNSCC), 754
- Healing of rickets, 381
- Health  
 disparities, 924  
 microbiota in, 1059–1060  
 outcomes, 201–202  
 regulation of CYP2R1 in, 354–355  
 “Health conscious” individuals, 40–41  
 Health-related quality of life, 318  
 Healthy volunteer bias, 25  
 Healthy volunteer selection bias, 17  
 Heart failure, 571, 575–578  
 Heavy alcohol consumers, 938  
 Hedgehog (HH), 954  
 signaling, 973–974  
 $\alpha$ -helices, 342  
*Helicobacter*, 1059  
*H. pylori*, 997–998, 1067  
 Heliotherapy, 996  
 Helix 12, 379  
 Helminths, 1060–1061  
 Helper cells, 1186–1188  
 Hematological malignancies  
 clinical applications of VDDs actions  
 against hematopoietic malignancies, 768–774  
 differentiation of myeloid leukemia cells by VDDs, 741–742  
 signaling and execution of monocytic differentiation induced by VDDs, 742–752  
 significance of basic and clinical studies of VDDs in hematopoietic malignancies, 774–775  
 transcription factors in VDD-induced differentiation, 752–755  
 VDD effects  
 on cell cycle and proliferation of human leukemia cells, 755–764  
 on cell survival and cell death, 764–765  
 on lymphoid lineage cells, 765–768  
 VDDs-induced progression of monocytic to macrophage differentiation, 751  
 Hematopoietic cell precursors, 741  
 Hematopoietic stem and progenitor cell (HSPC), 742  
 Hematopoietic stem cells (HSCs), 742, 766  
 Hematoxylin, 1222–1223  
 Hemoglobin A1c, 175  
 Hepatic cytochrome P450 family 2 subfamily R member 1 (CYP2R1), 571–572



- Hepatic diseases, 668  
Hepatic DNL, 560  
Hepatic hydroxylation, 568  
Hepatic macrophages, 562–563  
Hepatic steatosis, 559  
Hepatic stellate cells (HSCs), 560–561, 813  
Hepatitis B virus, 1012–1013  
Hepatitis C virus (HCV), 799, 1012  
Hepatocellular carcinoma (HCC), 559, 679–680, 764, 813, 1012–1013  
Hepatocyte growth factor (HGF), 861–863  
Hepatocytes, 559  
Hepatocytespecificablation of CD36, 560  
Hepcidin antibacterial protein (HAMP), 998  
HER2, 826  
Herceptin, 826  
Hereditary 1,25-dihydroxyVitamin D Resistant Rickets (HVDRR), 327–328, 359–360, 666  
alopecia, 387–388  
cellular basis of HVDRR, 365–373  
initial studies using cultured skin fibroblasts, 365–372  
studies in cell types, 372–373  
clinical features, 361–364  
alopecia, 364  
alopecia and rickets in children with HVDRR, 362f  
clinical and biochemical findings and distinguishing features, 361–363  
pathophysiology, 363–364  
compilation of, 366–371t  
extra-skeletal actions of vitamin D in HVDRR patients, 388–390  
asthma in HVDRR patients, 389–390  
autoimmune disease and cancer, 389  
cardiovascular system in HVDRR patients, 390  
extra-skeletal aspects of HVDRR, 388–389  
immune system in HVDRR patients, 389  
reproductive history of HVDRR patients, 390  
hypothesis regarding mechanism for spontaneous healing, 385–387  
spontaneous healing, 385  
studies of calcium absorption and puberty, 385–387  
mechanism of 1,25(OH)<sub>2</sub>D action relevant to HVDRR, 364–365  
regulation of gene expression by 1,25(OH)<sub>2</sub>D, 364–365  
vitamin D receptor, 364  
mutations, 373–382  
compound heterozygous mutations in VDR, 380  
dominant negative mutations, 380–381  
first description of genetic defect in nuclear receptor superfamily, 373–374  
HVDRR in dogs and cats, 381  
HVDRR without mutations in VDR, 381–382  
mutations causing premature termination of VDR, 374–377  
mutations in VDR, 381  
mutations in VDR DNA binding domain, 374  
mutations in VDR ligand-binding domain, 377–380  
therapy of HVDRR, 382–385  
calcium, 383–384  
cinacalcet, 384–385  
future therapy using rationally designed vitamin D analogs, 385  
prenatal diagnosis, 385  
vitamin D, 382–383  
VDDR-1's, VDDR-3 and HVDRR, 360t  
Hereditary hypophosphatemic rickets with hypercalciuria (HHRH), 292–293  
Hereditary nonpolyposis colon cancer (HNPCC), 860  
Hereditary PC (HPC), 938  
Heritability of serum 25(OH)D levels, 186  
Herpes, 1013  
Heterogeneity, 173–174  
Heterogeneous diseases, 1234  
Heterogeneous nuclear ribonucleoprotein (hnRNP), 360–361, 382  
Heterogenous disease, 917–918  
Heterogenous nuclear ribonucleoprotein C (HNRNPC), 314  
HFD. *See* High-fat diet (HFD)  
HGF. *See* Hepatocyte growth factor (HGF)  
HGPIN. *See* High-grade prostatic intraepithelial neoplasia (HGPIN)  
HH. *See* Hedgehog (HH)  
HHRH. *See* Hereditary hypophosphatemic rickets with hypercalciuria (HHRH)  
HHV-6. *See* Human herpes-virus 6 (HHV-6)  
HHV4. *See* Human herpesvirus 4 (HHV4)  
HHV5. *See* Human herpes virus 5 (HHV5)  
HIF. *See* Hypoxia-inducible factor (HIF)  
High dietary calcium, 317  
High energy of UVB, 1058–1059  
High selenium broccoli sprouts, 959  
High-dimensional data approaches to understanding VDR functions in prostate, 923–925  
cistronic approaches, 923–925  
transcriptomic approaches, 923  
High-fat diet (HFD), 560  
High-grade prostatic intraepithelial neoplasia (HGPIN), 917  
High-performance liquid chromatography (HPLC), 48–49, 631, 665, 1265–1266  
High-resolution peripheral QCT (HR-pQCT), 164, 233, 417, 482  
High-throughput techniques, 691  
His229Gln. *See* Histidine to glutamine at amino acid 229(His229Gln)  
His305Gln, 378  
His35Gln, 374  
Histidine ammonia-lyase (HAL), 191–192  
Histidine to glutamine at amino acid 229(His229Gln), 380  
Histomorphometry, 227  
Histone acetyltransferases (HATs), 978  
Histone deacetylase (HDAC), 770  
Histoplasmosis, 632  
HIV. *See* Human immunodeficiency virus (HIV); Immunodeficiency virus (HIV)  
HL-60 subclone, 695  
HL60 cells, 701, 746–747, 759  
HLA. *See* Human leukocyte antigen (HLA)  
HLA-DR. *See* Humanleukocyte antigen–antigen D related (HLA-DR)  
HLA-DRB gene, 1133  
HLA-DRB1 gene, 1134  
HLA-DRB1\*15, 1135  
haplotypes, 1133  
HLA-DRB1\*1501, 1133, 1168  
HLX. *See* H2. 0-like homeobox transcription factor (HLX)  
HNPCC. *See* Hereditary nonpolyposis colon cancer (HNPCC)  
hnRNP. *See* Heterogeneous nuclear ribonucleoprotein (hnRNP)  
HNRNPC. *See* Heterogenous nuclear ribonucleoprotein C (HNRNPC)  
HNSCC. *See* Head and neck squamous cell carcinoma (HNSCC)  
Hodgkin's disease, 633  
Holy Grail of evidence-based medicine, The, 577  
HOMA-IR. *See* Homeostatic model assessment-IR (HOMA-IR)  
Homeostatic model assessment-IR (HOMA-IR), 501  
Hominissuis, 667  
Homo sapiens, 14–15  
Homocysteine (HCY), 1153–1154  
Horizontal pleiotropy, 42  
Hormonal adipokines, 498–499  
Hormonal regulation of FOXP3 gene, 1140–1145  
Hormone replacement therapy (HRT), 416  
Hormone treatment, 1135–1136  
Host defense peptides (HDPs), 1017  
HOXA10, 754  
HOXB4, 754, 762–763  
HPC. *See* Hereditary PC (HPC)  
HPK1A. *See* Human keratinocytes (HPK1A)  
HPLC. *See* High-performance liquid chromatography (HPLC)  
HPP. *See* Hypophosphatasia (HPP)  
HPV. *See* Human papilloma virus (HPV)  
HR. *See* Hazard ratio (HR)  
HR gene. *See* Hairless gene (HR gene)  
HR-pQCT. *See* High-resolution peripheral QCT (HR-pQCT)  
HRAS genes, 292  
HRT. *See* Hormone replacement therapy (HRT)

- HSCs. *See* Hematopoietic stem cells (HSCs); Hepatic stellate cells (HSCs)
- HSPC. *See* Hematopoietic stem and progenitor cell (HSPC)
- HTLV-1-transformed lymphocytes, 634
- HTLV-I. *See* Human T cell lymphotropic virus type I (HTLV-I)
- hTNFtg. *See* Human tumor necrosis factor  $\alpha$  transgenic mouse (hTNFtg)
- Human alpha defensin 4 (HAD-4), 1017–1018
- Human beta defensin 2 (HBD2), 1017–1018
- Human biology, 14–15
- Human bronchial epithelial cell (HBEC), 1048
- Human cationic antimicrobial protein (hCAP-18), 799
- Human corticalbone, 229
- Human cytomegalovirus (HCMV), 1011–1013
- Human herpes virus 5 (HHV5), 1013
- Human herpes-virus 6 (HHV-6), 1146–1147
- Human herpes virus 4 (HHV4), 1013
- Human IH phenotype, 621
- Human immune response, serial link among, 465–466
- Human immunodeficiency virus (HIV), 1011–1012
- Human innate responses, calcifediol treatment of microbial disease to boost, 465–469
- Human keratinocytes (HPK1A), 688
- Human leukemia cells proliferation, VDDs effects on, 755–764
- Human leukocyte antigen (HLA), 1110–1111, 1133, 1167–1168
- Human osteoclast precursors, overexpression of TAF12 is sufficient to induce  $1,25(\text{OH})_2\text{D}_3$  hyperresponsivity in, 654–655
- Human papilloma virus (HPV), 1014
- Human studies, 1115–1116
- Human T cell lymphotropic virus type I (HTLV-I), 633, 688
- Human tumor necrosis factor  $\alpha$  transgenic mouse (hTNFtg), 1190
- Humanleukocyte antigen–antigen D related (HLA-DR), 475–476
- Humans acquire vitamin D, 1076
- Humoral adaptive immunity, 1035–1036
- Humoral hypercalcemia of malignancy, 687–688
- Hungry bone syndrome, 254, 315, 627–628
- Hungry bones, 383–384
- HVDRR. *See* Hereditary 1,25-dihydroxyvitamin D Resistant Rickets (HVDRR); Hypocalcemicvitamin D-resistant rickets (HVDRR)
- HVO. *See* Hypovitaminosis D osteopathy (HVO)
- HVOi, 228
- Hyaluronan (HA), 1212
- Hyaluronic acid (HA), 1217
- Hydrocephalus, 246–247
- Hydrogen peroxide cytotoxicity, 979
- 8-hydroxy-20-deoxyguanosine (8-OHdG), 885
- Hydroxyapatite crystals, 226
- Hydroxychloroquine, 641
- Hydroxyl radicals, 979–980
- 1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase), 250, 304, 327, 360–361, 389–390, 404, 412, 490, 568, 592, 627–628, 1131–1132
- deficiency, 363
- enzyme, 995–996
- 1 $\alpha$ -hydroxylase, 42
- 24-hydroxylase (24-OHase), 288, 403f, 568, 620, 634, 906–907
- activity, 372–373
- expression, 592
- inhibition of catabolic pathway of, 636–637
- 25-hydroxylase (25-OHase), 186–187, 1111
- deficiency, 363
- 1 $\alpha$ -hydroxylation of 25(OH)D, 592
- 24-hydroxylumisterol3 (24(OH)L<sub>3</sub>), 972–973
- Hydroxyproline excretion, 249
- 15-Hydroxyprostaglandin dehydrogenase (15-PGDH), 805
- 1 $\alpha$ -hydroxyvitamin D (1 $\alpha$ -OHD), 332, 476
- 24-hydroxyvitamin D, 149
- 1 $\alpha$ -hydroxyvitamin D<sub>3</sub> (1 $\alpha$ -OHD<sub>3</sub>), 629
- Hygiene hypothesis, 1060–1061
- Hyp cortical bone, 288
- Hyp osteoblasts, 287
- Hypercalcemia, 21, 263, 401, 405, 407, 466, 579–580, 629–630, 632–633, 638, 640, 1079–1081
- clinical manifestations of vitamin D toxicity, 638–641
- diagnosis of vitamin D toxicity, 639
- treatment of vitamin D toxicity, 639–641
- forms of endogenous vitamin D toxicity, 630–634
- forms of exogenous vitamin D toxicity, 625–630
- Hypercalcemia-1 (HCINF1), 401
- Hypercalcemia-2 (HCINF2), 401
- Hypercalciuria, 333, 620, 629–630
- Hyperhomocysteinemia, 1154
- Hyperkalemia, 306
- Hyperparathyroidism, 252, 305
- Hyperphosphatemia, 569, 599–600, 608, 625–626
- clinical symptoms of, 284
- hyperphosphatemia-mediated hypocalcemia, 314–315
- Hyperproliferation, 881–882
- Hyperproliferative skin disorders, treatment of, 1223–1224
- Hypertension, 2, 135, 193, 215, 570, 577–578, 619
- systemic inflammation conferring renal and cardiovascular protection
- independently of renal o and r systemic klotho levels, vitamin D control of, 606–609
- vitamin D and, 500–501
- Hypertrophic chondrocytes, 224
- Hypervitaminosis D, 401, 626–627, 667
- Hypocalcemia, 259, 303, 314–315, 329, 598–599
- due to accelerated skeletal mineralization, 315
- clinical manifestations of, 305–306
- development of osteomalacia and rickets, 306f
- differential diagnosis of, 306–312
- classification, 306
- disorders of parathyroid gland formation, 306–309
- disorders of parathyroid hormone production or secretion, 309–310
- hypocalcemia due to abnormalities of parathyroid hormone availability, 306
- hypocalcemia due to resistance to actions of parathyroid hormone, 310–311
- hypomagnesemia, 312
- parathyroid gland damage/ destruction, 310
- physiology of, 303–305
- biochemical changes induced by, 305
- parathyroid hormone in acute defense of serum calcium concentration, 304
- vitamin D in long-term maintenance of eucalcemia, 304–305
- treatment of, 316–319
- acute management, 316
- genetic forms of vitamin D–dependent rickets, 319
- hypoparathyroidism, 317–318
- long-term management, 316–317
- pseudohypoparathyroidism, 318
- Hypocalcemic disorders, 303, 625–626
- clinical manifestations of hypocalcemia, 305–306
- dietary calcium deficiency, 314
- differential diagnosis of hypocalcemia, 306–312
- classification, 306
- disorders of parathyroid gland formation, 306–309
- disorders of parathyroid hormone production or secretion, 309–310
- hypocalcemia due to resistance to actions of parathyroid hormone, 310–311
- hypocalcemia due to abnormalities of parathyroid hormone availability, 306
- hypomagnesemia, 312
- parathyroid gland damage/ destruction, 310
- genetic forms of vitamin D–dependent rickets, 313–314

- Hypocalcemic disorders (*Continued*)  
 hyperphosphatemia-mediated  
   hypocalcemia, 314–315  
 hypocalcemia due to accelerated  
   skeletal mineralization, 315  
 medical illness, 315–316  
 parathyroid hormone-independent  
   hypocalcemia, 312–313  
   neonatal hypocalcemia, 312  
   nutritional vitamin D deficiency,  
     312–313  
   vitamin D malabsorption, 313  
 physiology of hypocalcemia, 303–305  
   biochemical changes induced by, 305  
   parathyroid hormone in acute  
     defense of serum calcium  
     concentration, 304  
   vitamin D in long-term maintenance  
     of eucalcemia, 304–305  
 treatment of hypocalcemia, 316–319  
   acute management, 316  
   hypoparathyroidism, 317–318  
   long-term management, 316–317  
 Hypocalcemicvitamin D-resistant rickets  
   (HVDRR), 359–360  
 Hypomagnesemia, 306, 312, 315  
 Hypoparathyroidism, 306, 310, 317–318,  
   625–626  
 Hypophosphatasia (HPP), 235  
 Hypophosphatemia, 264, 295, 329,  
   363–364  
   clinical symptoms of, 284  
 Hypophosphatemic osteomalacia, 228  
 Hypophosphatemic rickets, 233–234,  
   285–290  
 Hypotonia, 247–248  
 Hypovitaminosis D, 253, 513–514,  
   667–668  
 Hypovitaminosis D osteopathy (HVO),  
   227–228  
 Hypoxia-inducible factor (HIF), 874
- I**  
 IBD. *See* Inflammatory bowel disease (IBD)  
 ICC. *See* Intraclass correlation (ICC)  
 Ichthyosis, 1218  
 ICMA. *See* Immunochemiluminometric  
   assay (ICMA)  
 ICSBP. *See* Interferon-regulatory factor 8  
   (IRF8)  
 ICU. *See* Intensive care unit (ICU)  
 Id2. *See* Inhibitor of differentiation-2 (Id2)  
 ID2/ID4 gene, 1152–1153  
 Id4. *See* Inhibitor of differentiation-4 (Id4)  
 Idiopathic hypercalciuria (IH), 620–621  
   vitamin D metabolic pathways and  
   activating and inhibiting factors  
   associated with kidney stone  
   formation, 621f  
 Idiopathic hypoparathyroidism, 309  
 Idiopathic infantile hypercalcemia (IIH),  
   401  
 IDL. *See* Intermediate-density lipoprotein  
   (IDL)  
 IFN- $\gamma$ . *See* Interferon- $\gamma$  (IFN- $\gamma$ )
- IFNA1 gene, 1135  
 IGF. *See* Insulin-like growth factor (IGF)  
 IGF binding proteins (IGFBPs), 696  
 IGF-1BPs. *See* Insulin-like growth factor  
   and binding proteins (IGF-1BPs)  
 IGF-binding protein-6 (IGFBP-6), 873  
 IGF-R-II. *See* Type II IGF receptor (IGF-R-  
   II)  
 IGFBP-3. *See* Insulin-like growth  
   factor-binding protein 3 (IGFBP-3)  
 IGFBP-6. *See* IGF-binding protein-6  
   (IGFBP-6)  
 IGFBPs. *See* IGF binding proteins (IGFBPs)  
 IGFR, 562–563  
 IGFR. *See* Insulin-like growth factor  
   receptor (IGFR)  
 IH. *See* Idiopathic hypercalciuria (IH)  
 IHH. *See* Indian hedgehog (IHH)  
 IIH. *See* Idiopathic infantile hypercalcemia  
   (IIH)  
 I $\kappa$ B. *See* Inhibitor of  $\kappa$ B (I $\kappa$ B)  
 Ikzf2 gene, 1130–1131, 1137–1139  
 IL. *See* Interleukin (IL)  
 IL2ragene, 1138  
 ILC. *See* Innate lymphoid cells (ILC)  
 ILC3. *See* Innate lymphoid cells (ILC3)  
 Ile314Ser. *See* Isoleucinetio serine at amino  
   acid 314 (Ile314Ser)  
 Iliac crest bone biopsies, 165  
 ILX23–7553, 764  
 Immediate release calcifediol (IRC), 462  
 Immortalized B lymphoblasts, 372–373  
 Immune cells, 953, 1049  
   vitamin D effect on, 801–802  
 Immune disease, epidemiological and  
   evidence supporting role of vitamin  
   D in, 1036  
 Immune disorders, 216–217  
 Immune effects of vitamin D, 476  
 Immune microenvironment and function  
   of APCs, actions of vitamin D on,  
   1043–1044  
 Immune modulation, 2  
 Immune regulation, 242–243  
 Immune response in tuberculosis, 1076  
 Immune suppression, 968–972  
 Immune system, 995, 1035, 1058,  
   1062–1065, 1109, 1114  
   in HVDRR patients, 389  
 Immune TME compartment, 957  
 Immune-mediated diseases, 669, 1057,  
   1114  
 Immune-mediated polyarthritis, 667  
 Immunity, 1250  
   effects of vitamin D on, 475–476  
   research, 1250  
   vitamin D and, 1091–1094  
 Immunoassay-based techniques, 665  
 Immunochemiluminometric assay  
   (ICMA), 639  
 Immunodeficiency virus (HIV), 1075  
 Immunoglobulin, 1036  
 Immunological methods, 48–49  
 Immunomodulation, 874–876  
   vitamin D and, 1015–1016
- Immunomodulatory effects, 874–878  
 Immunophenotypes, 1243  
 Immunoradio metric assay (IRMA), 639  
 Immunosuppressants, 1120–1121  
 Immunosuppressive cytokines, 972  
 Immunosuppressive drugs, 1067  
 Immunotherapy, 903  
 Impaired autophagic responses, 998–999  
 IMSCG. *See* International Multiple  
   Sclerosis Genetics Consortium  
   (IMSCG)  
 In vitro effects of vitamin D pathway in  
   breast cancer model systems, 845t  
 In vitro studies, 512–513  
 In vivo analyses of 1,25(OH) $_2$ D $_3$ actions,  
   921–922  
 In vivo animal models, 1038–1039  
 In vivo evidence for 1,25(OH) $_2$ Dregulation  
   of antibacterial innate immunity,  
   1002–1004  
 In vivo studies, 512–513, 839  
 Inadequate mineralization, 567–568  
 Incident fractures, 435–436  
   RCTs of supplemental vitamin D *vs.*  
   placebo on incident fractures in  
   general population, 448–450  
   systematic reviews and metaanalyses of  
   supplemental vitamin D and  
   calcium on, 446  
 Indian hedgehog (IHH), 224, 288  
 Individual clinical trials, results from,  
   533–539  
   diabetes prevention with active vitamin  
   D study, 538–539  
   large trials, 539  
   Tromsø study, 533–537  
   vitamin D and type 2 diabetes study,  
   537–538  
 Individual participant data (IPD), 1239  
   results from IPD metaanalysis, 541–542  
 Indoor work, 105  
 Indoor workers, 103–104  
 Induce osteomalacia (TIO), 282–283  
 Inducible nitric oxide synthase (iNOS),  
   805, 969  
 Induction of E-cadherin, 868–869  
 Inecalcitol, 768–769, 774  
 Infantile hypercalcemia, 401  
   clinical features, 404–405  
   diagnosis, 405  
   genetic basis of disease, 402–403  
   historical notes, 401–402  
   24-Hydroxylase and vitamin D  
   metabolism, 403f  
   pathophysiology of disease, 403–404  
   therapy, 405–407  
 Infants, 242, 248  
 Infectious diseases, 2, 668–669  
 Inflammasome activation, 1264  
 Inflammation and hormonal adipokines,  
   498–499  
 Inflammation parameters, 575  
 Inflammatory bowel disease (IBD), 2,  
   667–668, 797, 802, 859, 996,  
   1057–1062

- associations between serum 25(OH)D levels and clinical IBD severity, 1062t
- current treatments for, 1067–1068
- microbial interventions and IBD, 1068
- environment, 1059
- experimental models of, 1065–1067, 1066t
- vitamin D and microbiota, 1066–1067
- genetic factors, 1058–1059
- gut epithelium, commensals, and IBD, 1061–1062
- hygiene hypothesis, 1060–1061
- immune system and inflammatory
- bowel disease, 1062–1065
- microbiota in health and disease, 1059–1060
- vitamin D as treatment option for inflammatory bowel disease, 1068
- vitamin D hypothesis, 1061
- Inflammatory T helper 17 (Th17), 1263
- Inflammatory/inflammation, 670, 692, 797–803, 874–876, 921, 1012–1013, 1112
- cells, 797
- characteristics, 798–799
- inflammation-associated cancer, 803–813
- key factors in, 804–806
- vitamin D and, 806–813
- process, 692
- vitamin D and, 1091–1094
- vitamin D effects, 799–802
- in inflammation-associated breast cancer, 810
- in inflammation-associated colorectal cancer, 806–807
- in inflammation-associated hepatocellular cancer, 813
- in inflammation-associated pancreatic cancer, 811–812
- in inflammation-associated prostate cancer, 808–809
- in inflammatory diseases, 802–803
- Infliximab, 1067–1068
- Influenza virus, 1012
- Information bias, 41
- Inherited recessive disorders, 971
- Inhibitor of differentiation-2 (Id2), 1152
- Inhibitor of differentiation-4 (Id4), 1152
- Inhibitors of  $\kappa\text{B}$  (I $\kappa\text{B}$ ), 876
- Inkt. *See* Invariant natural killer T (Inkt)
- Innate immune responses, 1012
- Innate immune sensing of viruses, 1,25(OH)<sub>2</sub>D and, 1016–1017
- Innate immunity, 1264
- 1,25(OH)<sub>2</sub>D<sub>3</sub>, 766
- Innate lymphoid cells (ILC), 1065–1066
- Innate lymphoid cells (ILC3), 875
- Inorganic form, 281
- Inorganic phosphate (Pi), 281
- Inorganic phosphorus (Pi), 226
- Inorganic pyrophosphate (PPi), 226
- iNOS. *See* Inducible nitric oxide synthase (iNOS)
- Inositol 1,4,5-triphosphate (IP<sub>3</sub>), 746–747
- Institutes of Medicine (IOM), 12–13, 446, 568, 579
- Insufficiency, 117
- Insulin, 1111–1112
- secretion, 499–500, 511–513
- vitamin D and insulin sensitivity, 513–514
- Insulin and homeostasis model
- assessment (HOMA-IR), 513–514
- Insulin resistance (IR), 475, 499–500
- Insulin-like growth factor (IGF), 873
- IGF-1, 171, 499
- IGF-II, 696
- Insulin-like growth factor and binding proteins (IGF-1BPs), 499
- Insulin-like growth factor receptor (IGFR), 764
- Insulin-like growth factor-binding protein 3 (IGFBP-3), 218
- Insulin-responsive tissues, 513–514
- Intensive care unit (ICU), 466–467, 1004, 1091, 1259
- Interferon-inducible protein-10 (IP-10), 1018
- Interferon-regulatory factor 8 (IRF8), 752
- Interferon-stimulated genes (ISGs), 1012, 1018
- Interferon- $\gamma$  (IFN- $\gamma$ ), 698, 767, 799, 1076, 1092, 1189–1190
- IFN- $\gamma$ -activation, 927
- IFN- $\gamma$ -stimulated beta cell, 1112
- Interleukin (IL), 1112, 1208
- IL-1, 634, 995–996
- IL-1 $\beta$ , 561–562, 997
- IL-2, 1092
- IL-4, 1189–1190
- IL-6, 171, 498–499, 561–562, 927
- effects of IL-6 expression by osteoclast precursors, 654
- gene, 1134
- IL-10, 972, 1058, 1146, 1243
- 1,25(OH)<sub>2</sub>D<sub>3</sub> modulation of IL-10 cell production, 1147–1148
- gene, 1134
- transcripts, 1134
- IL-12, 1076
- IL-17, 1189–1190
- Intermediate-density lipoprotein (IDL), 218
- “Intermediate” phenotype, 404–405
- Internal standards, 81–82
- International Agency for Research on Cancer (IARC), 939–947
- International Commission on Illumination (CIE), 100
- International Multiple Sclerosis Genetics Consortium (IMSGC), 1172–1174
- International Osteoporosis Foundation (IOF), 4
- International Society for Heart and Lung Transplantation, 480
- International Units (IU), 13, 1094
- Intervention, 1265
- Interventional data, 1267–1270
- Interventional studies, 41–42, 827–832, 827t–828t
- association between vitamin D insufficiency and viral disease in, 1012–1015
- Intestinal calcium, 168
- Intestinal SCs (ISCs), 880
- Intestine, 162
- Intopyrophosphate (PPi), 291
- Intracerebral hemorrhage, 573–574
- Intraclass correlation (ICC), 186
- Intracrine calcitriol, 465
- Intracrine vitamin D pathways, 476
- Intraepithelial lymphocyte, 767
- Intravenous calcium gluconate, 316
- Intravenous immunoglobulin (IVIG), 1219
- Intravenous IRC (IV IRC), 463–464
- Intravenous therapy (IV therapy), 381, 383–384
- Invariant natural killer T (Inkt), 1063–1064
- Inverse supplement hypothesis, 123
- Inverse variance weighted (IVW), 202
- IOF. *See* International Osteoporosis Foundation (IOF)
- IOM. *See* Institutes of Medicine (IOM); US Institute of Medicine (IOM)
- Ionized calcium, 303
- IP-10. *See* Interferon-inducible protein-10 (IP-10)
- IPD. *See* Individual participant data (IPD)
- Ipilimumab, 903
- IQ. *See* 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)
- IR. *See* Insulin resistance (IR)
- IRC. *See* Immediate release calcifediol (IRC)
- IRC in ARI including COVID-19, use of, 467–469
- IRF8. *See* Interferon-regulatory factor 8 (IRF8)
- IRI. *See* Ischemia reperfusion (IRI); Renal ischemia reperfusion (IRI)
- IRMA. *See* Immunoradio metric assay (IRMA)
- Iroquois homeobox transcription factor 3 (IRX3), 753
- Iroquois homeobox gene 5 (IRX5), 762–763
- IRX3. *See* Iroquois homeobox transcription factor 3 (IRX3)
- IRX5. *See* Iroquois homeobox gene 5 (IRX5)
- Ischemia reperfusion (IRI), 1265–1266
- Ischemic stroke, 573–574
- Ischemic heart disease, 571, 573–574
- ISCs. *See* Intestinal SCs (ISCs)
- ISGs. *See* Interferon-stimulated genes (ISGs)
- Isobaric metabolites, 665
- Isoleucine to serine at amino acid 314 (Ile314Ser), 377
- Isomeric metabolites, 665
- IU. *See* International Units (IU)
- IV IRC. *See* Intravenous IRC (IV IRC)
- IV therapy. *See* Intravenous therapy (IV therapy)



- IVIG. *See* Intravenous immunoglobulin (IVIG)
- IVW. *See* Inverse variance weighted (IVW)
- J**
- JAK/STAT pathway, 752
- Japan Public Health Center-based Prospective Study, 720
- JmJC. *See* Jumonji C (JmJC)
- JMJD. *See* Jumonji C domain-containing protein (JMJD)
- JMJD1A. *See* Jumonjido domain containing 1A (JMJD1A)
- JNK. *See* c-Jun N-terminal kinase (JNK)
- Jumonji C (JmJC), 868–869
- Jumonji C domain-containing protein (JMJD), 868–869
- Jumonjido domain containing 1A (JMJD1A), 883
- K**
- Kallikrein-related peptidase 10 (KLK10), 192
- Kaplan–Meier survival estimates, 479f
- KCs. *See* Kupffer cells (KCs)
- KDIGO. *See* Kidney Disease Improving Global Outcomes (KDIGO)
- Kenny–Caffey syndrome type 2, 309
- Keratinocyte stem cells (KSC), 388
- Keratinocytes, 741, 759, 1002, 1263
- Ketoconazole, 641
- KI mice. *See* Knock-in mice (KI mice)
- Kidney Disease Improving Global Outcomes (KDIGO), 462, 480
- Kidney(s), 567–568
- stone disease, 619
- transplant patients, 478–479
- vitamin D supplementation and kidney stone risk, 622
- Kinase inhibitors, 771
- Kinase suppressors of RAS-1 and RAS-2 (KSR1–2), 751
- KL mutations, 402
- KLF4. *See* Krüppel-like factor4 (KLF4)
- Klinefelter syndrome, 342–344
- KLK10. *See* Kallikrein-related peptidase 10 (KLK10)
- KLK6 gene, 692
- Klotho
- axis, 603–606
- gene, 294
- klotho-derived peptides, 605
- klotho-null mouse, 294
- Knee X-rays with typical signs of rickets, 236f
- Knock-in mice (KI mice), 657
- Knockout mice, 225
- Knockout models in experimental animals, 568
- Koreancohort study, 560
- Krasmutant, 956
- Krüppel-like factor4 (KLF4), 608
- KSC. *See* Keratinocyte stem cells (KSC)
- KSR1–2. *See* Kinase suppressors of RAS-1 and RAS-2 (KSR1–2)
- Kupffer cells (KCs), 560–561
- L**
- L-carnitine, 959
- LAB. *See* Lactic acid bacteria (LAB)
- Lactate dehydrogenase (LDH), 1103
- Lactation, 626
- course of, 242
- pregnancy and, 254
- Lactic acid bacteria (LAB), 877
- LADA. *See* Latent autoimmune diabetes in adults (LADA)
- LAG-3. *See* Lymphocyte activation gene 3 protein (LAG-3)
- LAMP-1. *See* Lysosomal associated membrane protein-1 (LAMP-1)
- Langerhans cells (LCs), 1049
- Lap band. *See* Adjustable gastric band
- Laparoscopic surgery, 162
- Large cross-sectional study, 515–516
- Large randomized controlled trials of supplemental vitamin D on fractures, 444t–445t
- Large vitamin D supplementation trials in general populations, cardiovascular events in, 577–578
- Late neonatal hypocalcemia, 312
- Latent autoimmune diabetes in adults (LADA), 1116
- Latent MTB infection (LTBI), 1075
- Latitude, 1045
- Latitude cover, 34
- Latitude–skin color mismatch, 115–116
- LBD. *See* Ligand binding domain (LBD)
- LC. *See* Low calcium (LC)
- LC-MS/MS. *See* Liquid chromatography tandem mass spectrometry (LC-MS/MS)
- LCA. *See* Litochoic acid (LCA)
- LCFAs. *See* Long-chain fatty acids (LCFAs)
- LCMV. *See* Lymphocytic choriomeningitis virus (LCMV)
- LCs. *See* Langerhans cells (LCs)
- LDH. *See* Lactate dehydrogenase (LDH)
- LDL. *See* Low-density lipoprotein (LDL)
- LDL receptor adaptor protein 1 (LDLRAP1), 751
- LDL receptor-related protein (LRP), 861
- LDLR gene, 187–189, 562–563
- LDLRAP1. *See* LDL receptor adaptor protein 1 (LDLRAP1); Lipoprotein receptor adaptor protein 1 (LDLRAP1)
- Leflunomide, 1119
- Left ventricular ejection fraction (LVEF), 575–576
- Left ventricular end-diastolic diameter (LVEDD), 575–576
- Left ventricular hypertrophy (LVH), 587–588
- Length of stay (LOS), 1103
- Lentivirus, 1012
- Leptin, 171–172, 499
- Lethargy, 667
- Leucine-rich repeat-containing G protein–coupled receptor (LGR), 861
- Leukemia stem cells, 742
- Leukemic stem cell (LSC), 752
- Leukocytes, 1076
- mRNA expression, 1268–1269
- Lewis lung carcinoma cells, 687
- LGR. *See* Leucine-rich repeat-containing G protein–coupled receptor (LGR)
- LH. *See* Luteinizing hormone (LH)
- Lifestyle factors associated with reduced sun exposure, 105
- Ligand binding domain (LBD), 363–364, 769, 977
- mutations in VDR ligand-binding domain, 377–380
- schematic illustration of, 377f
- Light skin pigmentation, 1260
- Light–not UVB, effect of exposure to, 88
- Limit of quantification (LOQ), 82
- LINC00536. *See* Long intergenic nonprotein coding RNA 536 (LINC00536)
- LINC01122. *See* Long intergenic nonprotein coding RNA 1122 (LINC01122)
- Linear nevus sebaceous syndrome (LNSS), 285t, 292
- LIP, 600–601
- LIPCg ene, 187–189
- LIPG gene, 187–189
- Lipid parameters, 574–575
- Lipid profiles, 501
- Lipid source, coingestion with, 125–126
- Lipopolysaccharides (LPS), 876–877, 995–996, 1094
- Lipoprotein receptor adaptor protein 1 (LDLRAP1), 812
- Lipoprotein receptor–related protein 5/6 (LPR5/6), 605, 805–806
- Lipoproteins, 1262
- Liposomes, 562–563
- Lipoteichoic acid, 1262
- Liquid chromatography, 47, 77–78
- Liquid chromatography tandem mass spectrometry (LC-MS/MS), 404, 665, 1265–1266
- Listeria monocytogenes*, 1002, 1263
- Litochoic acid (LCA), 769
- derivatives, 769
- Liver, 559–560
- disease, 265
- fibrosis, 562
- inflammation, 560–562
- metabolism and disease
- vitamin D/VDR signaling and antiinflammatory responses in liver, 561–562
- vitamin D/VDR signaling in liver fibrosis and NASH, 562–563
- of vitamin D/VDR signaling on chronic liver diseases, 561f
- vitamin D deficiency in patients with liver failure, 477

- vitamin D/VDR signaling in liver fibrosis and NASH, 562–563  
 Liver X receptors (LXR), 560, 1210  
 LKB1 mutation, 938  
 LL-37, 466  
 LMICs. *See* Low-and middle-income countries (LMICs)  
 lncRNA. *See* Long non-coding RNA (lncRNA)  
 LNSS. *See* Linear nevus sebaceous syndrome (LNSS)  
 Local metabolism of vitamin D in mammary gland, 836–837  
 Long bones, 224  
 Long intergenic nonprotein coding RNA 1122 (LINC01122), 190  
 Long intergenic nonprotein coding RNA 536 (LINC00536), 190  
 Long non-coding RNA (lncRNA), 189, 691, 870, 975–976  
 Long-chain fatty acids (LCFAs), 560  
 Long-latency disorders, 17  
 Long-term eucalcemia, 304  
 Long-term maintenance of eucalcemia, vitamin D in, 304–305  
 Long-term transplant recipients, vitamin D deficiency in, 478–480  
     Kaplan–Meier survival estimates in cardiac transplant recipients, 479f  
 Longitudinal cohort studies, 516–525  
 Longitudinal observational studies, 511  
 LOQ. *See* Limit of quantification (LOQ)  
 LOS. *See* Length of stay (LOS)  
 Loss-of-function, 309  
 Low bone density, 342–344  
 Low bone mass, 237  
 Low calcium (LC), 636  
 Low dairy product intake, 258  
 Low dietary calcium, graphic representation of synergistic effects of, 245f  
 Low vitamin D status, 670  
     prevalence of, 135–136  
 Low-and middle-income countries (LMICs), 244–245  
 Low-calcium diets, 413  
 Low-density lipoprotein (LDL), 591  
 Low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), 805–806  
 Low-incidence areas, 1059  
 Lowe syndrome, 284–285  
 LPA. *See* Lysophosphatidic acid (LPA)  
 LRP5/6. *See* Lipoprotein receptor-related protein 5/6 (LRP5/6)  
 LPS. *See* Lipopolysaccharides (LPS)  
 LRP. *See* LDL receptor-related protein (LRP)  
 LRP5/6. *See* Low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6)  
 LS. *See* Lumbar spine (LS)  
 LSC. *See* Leukemic stem cell (LSC)  
 LSCC. *See* Squamous cell carcinoma of the lung (LSCC)  
 LTBI. *See* Latent MTB infection (LTBI)  
 LUAD. *See* Adenocarcinoma of lung (LUAD)  
 Lumbar spine (LS), 480–481  
 Lumisterol (L<sub>3</sub>), 972–973, 1221  
 Lung cancer, 727, 901. *See also* Breast cancer (Bca); Skin cancer  
     classification and treatment, 902f  
     treatment varies by stage at diagnosis and molecular features of tumor, 903f  
     vitamin D, 903–904  
         and chemoprevention of NSCLC, 904–905  
         combining vitamin D with immunotherapeutics, 908–909  
         combining vitamin D with targeted therapeutics, 907–908  
         in management of advanced lung cancer, 905–909  
         in management of early-stage lung cancer, 905  
         strategies to maximize vitamin D signaling in, 909–910  
 Lung development, 1235–1236, 1249  
     potential mechanisms, 1248  
 Lung function, 1244–1245  
     and respiratory conditions, 216  
 Lung mesenchymal cell proliferation, 1235  
 Luteinizing hormone (LH), 840  
 LVEDD. *See* Left ventricular end-diastolic diameter (LVEDD)  
 LVEF. *See* Left ventricular ejection fraction (LVEF)  
 LVH. *See* Left ventricular hypertrophy (LVH)  
 LXR. *See* Liver X receptors (LXR)  
 Lymphocyte activation gene 3 protein (LAG-3), 954  
 Lymphocytes, 745, 1036  
 Lymphocytic choriomeningitis virus (LCMV), 1021  
 Lymphoid lineage cells, VDDs effects on, 765–768  
     B lymphocytes, 767–768  
     T lymphocytes, 767  
 Lymphoma, 633–634, 639  
     decreased clearance of 1,25-dihydroxyvitamin D, 634  
 Lynch syndrome, 860  
 Lys45 glutamutation, 374  
 Lysophosphatidic acid (LPA), 283–284  
 Lysophospholipids, 192  
 Lysosomal associated membrane protein-1 (LAMP-1), 1019
- ## M
- Macrophage colony-stimulating factor, 698, 1018  
 Macrophage inflammatory protein-1 beta (MIP-1β), 1018  
 Macrophages, 476, 745, 927, 1036, 1092, 1185–1186  
 Magnesium, 263, 295, 312  
     deficiency, 317  
     role in vitamin D, 126  
     supplementation, role of, 316  
 Magnetic resonance imaging (MRI), 1139, 1175, 1177–1178  
 Major histocompatibility complex (MHC), 1058, 1109, 1146  
     MHC II, 1076  
 Malakoplakia, 632–633  
 Malnutrition, 303–304  
 Mammalian target of rapamycin (mTOR), 598, 998–999  
 Mammalian target of rapamycin complex 1 (mTORC1), 982  
 Mammary carcinogenesis, VDR effect ablation on, 841  
 Mammary cell lineages, 827  
 Mammary gland, local metabolism of vitamin D in, 836–837  
 Man-fish, vitamin D in food farmed by, 84–87  
 Mandatory fortification  
     combined policies of, 131  
     of margarine and spreadable fat, 87  
     with vitamin D, 144  
     of wheat, 148  
 MAPK. *See* Mitogen-activated protein kinase (MAPK)  
 MAR. *See* Mineral apposition rate (MAR)  
 Marine omega-3 fatty acids, 88  
 Markers, 742  
 Marrow stromal cells derived from P62<sup>P394L</sup> knock-in mice and P62<sup>P392L</sup> Paget's patients hyperresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub> and display enhanced RANKL production in response to 1,25(OH)<sub>2</sub>D<sub>3</sub>, 657  
 MARRS. *See* Membrane-associated rapid response steroid (MARRS)  
 MART-10 (vitamin D analog), 687  
 MAs. *See* Metaanalyses (MAs)  
 MasR. *See* Mass receptor (MasR)  
 Mass receptor (MasR), 1092  
 Mass spectroscopy (MS), 404  
 Massive lymphadenopathy, 632–633  
 Mast cell, 870–871, 972  
 Master transcription factors, 754  
 Maternal antibodies, 1059–1060  
 Maternal vitamin D  
     deficiency, 243  
     metabolism, 501–502  
 Maternally expressed gene 3 (MEG3), 872  
 Matrix extracellular phosphoglycoprotein (MEPE), 293  
 Matrix metalloproteinases (MMPs), 805, 1077, 1219  
     secretion, 1217  
 Matrix vesicles (MVs), 226  
 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, 1214  
 Matrixglia Protein (MGP), 126, 569  
 Mature DC (mDC), 1186  
 Maxacalcitol, 909, 1222–1223  
 Maximum tubular reabsorption rate for phosphate (TmP), 282

- MBDP. *See* Metabolic bone disease of prematurity (MBDP)
- MBP. *See* Myelin basic protein (MBP)
- MC1R gene, 185–186, 1131
- MCF-7 cells, 693–694
- MCF10A-ras. *See* RAS-transformed MCF10A (MCF10A-ras)
- MCM 2e7. *See* Mini chromosome maintenance 2e7 (MCM 2e7)
- MCP-1. *See* Monocyte chemoattractant protein-1 (MCP-1)
- MDA5. *See* Melanoma differentiation-associated gene 5 (MDA5)
- mDC. *See* Mature DC (mDC)
- MDM2 gene, 980
- MDR disease. *See* Multidrug-resistant disease (MDR disease)
- MDS. *See* Myelodysplastic syndrome (MDS)
- MDSCs. *See* Myeloid-derived suppressor cells (MDSCs)
- Mean therapy, 419
- Measles virus (MV), 651  
infection of osteoclast precursors induces TAF12, 653
- Measles virus nucleocapsid protein gene (MVNP), 651, 653  
MVNP-transfected NIH3T3 cells, 656  
in OCL precursors, effects of transduction of TAF12 antisense construct on OCL formation in marrow cultures from PD patients expressing, 655
- Measurement error, 32
- Meat mushrooms, vitamin D in food farmed by, 84–87
- Mechanisms of action of vitamin D, 498
- Medial calcification of arterial vessels, 459–460
- Medical illness, 315–316  
medications, 315–316
- Medications, 315–316  
usage, 1261
- Mediterranean diet, 448
- Meet status gap, dietary recommendations to, 136
- Meeting nutritional requirements, sources of vitamin D and role of food in, 136–137
- MEG3. *See* Maternally expressed gene 3 (MEG3)
- Megalin, 492–493
- Melanin, 968
- Melanocytes, 973–974
- Melanoma, sun avoidance because of concern about, 103
- Melanoma differentiation-associated gene 5 (MDA5), 1016–1017
- MELD. *See* Model for end-stage liver disease (MELD)
- Membrane (M), 1091
- Membrane-associated rapid response steroid (MARRS), 598, 749–750
- Memory cells, 798–799
- Memory T cells from synovial fluid, 1188, 1188f
- Mendelian randomization studies (MR studies), 42–43, 193, 201, 353–354, 428, 525–526, 571–574, 682, 730–731, 1174–1175, 1238  
establish vitamin D insufficiency as causal for MS, 1132–1133  
of genetically determined 25(OH)D levels and risk of MS, 1173t
- MEPE. *See* Matrix extracellular phosphoglycoprotein (MEPE)
- MERS-CoV. *See* Middle East respiratory syndrome coronavirus (MERS-CoV)
- Messenger RNA (mRNA), 412, 1083
- MET. *See* Methionine (MET)
- Metaanalyses (MAS), 145–148, 423–425, 570–571
- Metabolic pathways, 692  
rickets of metabolic bone disease of prematurity, 263–265  
switch, 846
- Metabolic bone disease of prematurity (MBDP), 263  
antenatal and postnatal risk factors of, 263t
- Metabolic syndrome (MetS), 135, 167, 489, 497–501, 619  
dyslipidemia and nonalcoholic fatty disease of liver, 501  
inflammation and hormonal adipokines, 498–499  
insulin resistance, insulin secretion, and glycemia, 499–500  
vitamin D and hypertension, 500–501  
importance of early-life vitamin D status for later obesity and subsequent risks of, 501–503
- Metabolism and role of vitamin D on skeletal health, 663–665
- Metabolomics, 1270  
of vitamin D, 1264–1265
- Metalloproteinase (MMP2), 953
- Metallothionein, 979–980
- Metastasis, 686–687
- Metastasis-associated protein-3 (MTA-3), 697–698
- Methionine (MET), 1130–1131, 1152  
cycle, 1149–1155  
enhancement, 1153–1155
- Methionine–homocysteine cycle, 1153f
- Methotrexate (MTX), 1216–1217
- 2-Methylene-19-nor-(20S)-1,25(OH)<sub>2</sub>D<sub>3</sub> (2MD), 427
- 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), 904
- Methyltransferase (MTR), 1154
- MetS. *See* Metabolic syndrome (MetS)
- Mexico, vitamin D status in, 49
- MG-63 osteosarcoma cell line, 688–690
- MGAT1 transcripts, 1147
- MGP. *See* Matrix gla Protein (MGP)
- MHC. *See* Major histocompatibility complex (MHC)
- Microalgae, 125
- Microbe-specific regulatory T cells, 1060
- Microbial disease to boost human innate and adaptive immune responses, calcifediol treatment of, 465–469
- Microbial infections, 457  
and serum 25-hydroxyvitamin D (25(OH)D) status of host, 466–467
- Microbial interventions and IBD, 1068
- Microbial membranes, 466
- Microbial transplants, 1059
- Microbiome, 876–877
- Microbiota, 1057, 1060, 1066–1067  
in health and disease, 1059–1060
- Microbubbles, 562–563
- Micrometer-scale variations, 229
- Micronutrient, 1240  
coingestion with another, 126
- MicroRNA-145 (miR-145), 607–608
- MicroRNAs (miRNAs), 691, 867  
miR-22, 873  
miR-27b, 867  
miR-298, 867  
miR-346, 867
- Microsatellite instability (MSI), 860
- Middle East, vitamin D status in, 55, 59t–60t
- Middle East respiratory syndrome coronavirus (MERS-CoV), 608
- Migrants, 105–107
- Migration, 66, 105–107
- Milk, vitamin D in food farmed by, 84–87
- Mineral apposition rate (MAR), 228
- Mineral metabolism, 604–605
- Mineralization, 226
- Mineralocorticoid receptor antagonists, 575
- Minichromosome maintenance 2e7 (MCM 2e7), 757–758
- Minigastric bypass, 162
- Minimal erythematous dose, 100
- MIP-1β. *See* Macrophage inflammatory protein-1 beta (MIP-1β)
- miR-145. *See* MicroRNA-145 (miR-145)
- miR106b, 609
- miRNAs. *See* MicroRNAs (miRNAs)
- MIS. *See* Mullerian-inhibiting substance (MIS)
- Mismatch repair, 971
- Mitochondrial DNA (mtDNA), 1264
- Mitochondrial dysfunction, 1264
- Mitogen-activated protein kinase (MAPK), 746–747  
pathway, 747f, 749–751
- Mitogen-activated protein kinase phosphatase (MPK), 1243
- Mixed toxopherols, 959
- MLH1 mutation, 938
- MMP. *See* Mucous membrane pemphigoid (MMP)
- MMP2. *See* Metalloproteinase (MMP2)
- MMPs. *See* Matrix metalloproteinases (MMPs)
- MMR. *See* Mortality rate ratio (MMR)

- MMTV-PyMT mice. *See* Polyoma Virus middle T antigen in the mammary gland (MMTV-PyMT mice)
- Model for end-stage liver disease (MELD), 477
- Molecular  
biology studies, 1212–1213  
genetics of CYP24A1 mutations, 402–403  
molecular heterogeneity of human breast cancer, 826
- Monocyte 1,25(OH)<sub>2</sub>D production, 596
- Monocyte chemoattractant protein-1 (MCP-1), 1018
- Monocyte-specific esterase (MSE), 742
- Monocyte–macrophage 1,25(OH)<sub>2</sub>D synthesis, 595
- Monocytes, 476, 745, 927, 1130
- Monocytic differentiation induced by VDDs, signaling and execution of, 742–752
- Mononucleosis, 1013
- Monozygotic twins (MZ twins), 186
- Month-of-birth effect, 1131
- Mortality, 2, 19–20  
risk and aging, 217–218
- Mortality rate ratio (MMR), 1094
- Mouse Lewis lung carcinoma xenograft model, 909
- Mouse models, 602  
of polycystic kidney disease, 602–603  
of RA, 1189–1190
- Mouse OCLs inhibits bone destruction and bone remodeling, deleting TAF12 in, 657–658
- Mouse organoids, 881
- MPK. *See* Mitogen-activated protein kinase phosphatase (MPK)
- MR studies. *See* Mendelian randomization studies (MR studies)
- MRI. *See* Magnetic resonance imaging (MRI)
- mRNA. *See* Messenger RNA (mRNA)
- MRP. *See* Multidrug resistance–associated protein (MRP)
- MS. *See* Mass spectroscopy (MS); Multiple sclerosis (MS)
- MSE. *See* Monocyte-specific esterase (MSE)
- MSH2 mutation, 938
- MSI. *See* Microsatellite instability (MSI)
- MTA-3. *See* Metastasis-associated protein-3 (MTA-3)
- MTB. *See* *Mycobacterium tuberculosis* (MTB)
- mtDNA. *See* Mitochondrial DNA (mtDNA)
- MTHFR. *See* N,N-methylene-tetrahydrofolate reductase (MTHFR)
- mTOR. *See* Mammalian target of rapamycin (mTOR)
- MTR. *See* Methyltransferase (MTR)
- MTX. *See* Methotrexate (MTX)
- Muc. *See* Mucin (Muc)
- Mucin (Muc), 885
- Mucous membrane pemphigoid (MMP), 1218–1219
- Mullerian-inhibiting substance (MIS), 378
- Multicenter and global studies using central laboratory facility, 66
- Multidrug resistance–associated protein (MRP), 877
- Multidrug-resistant disease (MDR disease), 1083–1084
- Multifactorial diseases, 797
- Multiple sclerosis (MS), 69, 216, 1130, 1167–1168  
CD4<sup>+</sup>FOXP3<sup>+</sup>T regulatory cells and hormonal regulation of FOXP3, 1137–1145  
1,25(OH)<sub>2</sub>D<sub>3</sub> supports protective pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cell differentiation, 1138–1139  
androgens and estrogens support protective pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cell differentiation, 1139–1140  
discovery of CD4<sup>+</sup>Foxp3<sup>+</sup>Treg cells and dysfunction in MS, 1137–1138  
hormonal regulation of FOXP3 gene, 1140–1145  
sexual dimorphisms implicate hormonally regulated, CD4<sup>+</sup>T cell intrinsic protective mechanism, 1137  
descriptive epidemiology, 1168  
25-hydroxyvitamin D and, 1170–1171  
interplay between vitamin D and CD4<sup>+</sup> type 1 regulatory T cells in MS, 1145–1149  
1,25(OH)<sub>2</sub>D<sub>3</sub> as potential CD4<sup>+</sup>Tr1 cell inducer in MS patients, 1148–1149  
1,25(OH)<sub>2</sub>D<sub>3</sub> modulation of IL-10 and CD4<sup>+</sup>Tr1 cell production, 1147–1148  
CD4<sup>+</sup>Tr1 cells and MS, 1146–1147  
IL-10, CD4<sup>+</sup>Tr1 cell discovery and characteristics, 1146  
interplay between 1,25(OH)<sub>2</sub>D<sub>3</sub> and CD4<sup>+</sup>Tr1 cells in MS, 1149
- MS sexual dimorphism, 1134–1137, 1136t  
F:M incidence data over time and in transsexual individuals suggests hormone–gene interactions influence female MS risk, 1135–1136  
female–male differences provide insight into MS etiology, 1134–1135  
pregnancy studies suggest hormonally triggered reversible mechanism supporting reproductive success triggers MS remissions, 1136–1137  
oligodendrocytes, DNA methylation, and methionine cycle, 1149–1155  
1,25(OH)<sub>2</sub>D<sub>3</sub> supports pCD4<sup>+</sup>Foxp3<sup>+</sup>Treg cells promote myelin repair and replacement, 1150  
1,25(OH)<sub>2</sub>D<sub>3</sub>, BHMT gene, betaine, and methionine cycle enhancement, 1153–1155
- redox imbalance could undermine 25-hydroxyvitamin D-1 alpha hydroxylase enzyme activity, 1151–1152
- VDR signaling enhances methionine cycle, DNA methylation, and oligodendrocyte precursor cell differentiation, 1152–1153
- VDR signaling enhances oligodendrocyte differentiation and myelin synthesis, 1150–1151
- vitamin D system supports oligodendrocyte myelin repair and replacement, 1149–1150
- pediatric onset MS, 1178–1179
- randomized controlled clinical trials of vitamin D and MS outcomes, 1176t
- reflections on vitamin D<sub>3</sub> supplementation trials in MS, 1155–1156
- vitamin D, race, and MS risk, 1175
- vitamin D and, 1131–1134, 1168–1169  
early genetic support for vitamin D insufficiency in MS pathogenesis, 1131–1132  
mendelian randomization studies establish vitamin D insufficiency as causal for MS, 1132–1133  
vitamin D provides epigenetic insights into MS pathogenesis, 1133–1134  
vitamin D and MS disease activity and progression, 1175–1178  
conversion from CIS to MS, 1175–1177  
vitamin D and risk of MS, 1169–1175  
dietary intake of vitamin D and MS risk, 1169  
sun exposure and MS risk, 1169
- Murine models, 1049
- Muscle mass, loss of, 171
- Muscle tissue, 639
- Musculoskeletal health, 2  
determining optimal serum 25-hydroxyvitamin D concentrations for, 415–416
- Musculoskeletal outcomes, 215–216
- Mushrooms, 86
- Mutated CYP24A1 in vitamin D metabolism, 403–404
- Mutations, 373–382  
causing premature termination of VDR, 374–377  
compound heterozygous mutations in VDR, 380  
dominant negative mutations, 380–381  
first description of genetic defect in nuclear receptor superfamily, 373–374  
HVDRR in dogs and cats, 381  
HVDRR without mutations in VDR, 381–382  
in VDR DNA binding domain, 374  
in VDR ligand-binding domain, 377–380



- Mutations (*Continued*)  
 in VDR, 381  
 MV. *See* Measles virus (MV)  
 MVNP. *See* Measles virus nucleocapsid protein gene (MVNP)  
 MVs. *See* Matrix vesicles (MVs)  
 myCAFs. *See* Myofibroblasts CAFs (myCAFs)  
 Mycobacteria, 668–669  
*Mycobacterium avium*, 632, 667  
*Mycobacterium avium paratuberculosis*, 1059  
*Mycobacterium bovis*, 1075  
*Mycobacterium* infection, 670, 1271  
*Mycobacterium tuberculosis* (MTB), 248, 632, 799, 927, 995–996, 1075  
 influence of vitamin D on immune response to MTB, 1076–1079, 1077f  
 studies with MTB infection as end point, 1081–1082  
 intervention studies, 1082  
 observational studies, 1081  
 Mycophenolatemofetil (MMF), 1119  
 Myelin basic protein (MBP), 1149–1150  
 Myelin synthesis, 1150–1151  
 Myelocytosis, 248  
 Myelodysplastic syndrome (MDS), 768  
 Myelofibrosis, 752  
 Myeloid cells, 746  
 Myeloid leukemia cells differentiation by VDDs, 741–742  
 Myeloid metaplasia, 248  
 Myeloid progenitor cells, 372–373  
 Myeloid-derived suppressor cells (MDSCs), 953  
 Myelopoiesis, 742  
 Myoblasts, 741  
 Myocardial infarction, 570–571, 573–574, 577–578  
 Myofibroblasts CAFs (myCAFs), 953  
 Myogenic factor 5 promoter, 496  
 MZ twins. *See* Monozygotic twins (MZ twins)
- N**  
 N,N-methylene-tetrahydrofolate reductase (MTHFR), 1154  
 N-acetylgalactosaminyltransferase 2 (GALNT2), 190  
 N-acetyltransferase 2 (NAT2), 872  
 N-BPs. *See* Nitrogen-containing BPs (N-BPs)  
 N-nitroso-tris-chloroethylurea (NTCU), 904  
 NAD. *See* Nicotinamide adenine dinucleotides (NAD)  
 NADSYN1/DHCR-7, 1171–1172  
 NAFLD. *See* Nonalcoholic fatty liver disease (NAFLD)  
 Nail psoriasis treatment, 1216  
 Naïve T cells, 801, 1037–1038, 1037f  
*Nannochloropsis oceanica*, 125  
 Nanomedicine, 562–563  
 NaPi-IIc. *See* Sodium-dependent phosphate cotransporter (NaPi-IIc)
- Narrow band UV-B phototherapy, 1216–1217  
 NASH. *See* Nonalcoholic steatohepatitis (NASH)  
 NAT2. *See* N-acetyltransferase 2 (NAT2)  
 National Adult Nutrition Survey, 138–142  
 National Comprehensive Cancer Network (NCCN), 901–902  
 National Health and Nutrition Examination Survey (NHANES), 17, 33, 49, 135–136, 515–516  
 National Health and Nutrition Examination Survey III (NHANES III), 680–681  
 National Heart, Lung, and Blood Institute (NHLBI), 349  
 National Institute of Standards and Technology (NIST), 47, 666  
 National Institutes of Health (NIH), 24, 162, 537–538, 947  
 National Kidney Foundation, The (NKF), 609  
 National Multiple Sclerosis Society, 22  
 National nutrition surveys, 123  
 Natural content of vitamin D<sub>3</sub>, 88  
 Natural foods, 626–627  
 Natural health products (NHPs), 126–127  
 Natural killer cells (NK cells), 798–799, 1002, 1019, 1186–1187  
 1,25(OH)<sub>2</sub>Dand, 1002, 1019  
 Natural killer T cells (NKT cells), 1186–1187  
 Natural product number (NPN), 126–127  
 Natural sources, 1061  
 NBT. *See* Nitroblue tetrazolium (NBT)  
 NC. *See* Normal calcium (NC)  
 NCCN. *See* National Comprehensive Cancer Network (NCCN)  
 Necrobiotic xanthogranuloma, 633  
 Neonatal hypocalcemia, 312  
 Neoplastic  
 breast cells/tissues, 832–839  
 disease, 825–826  
 NEP. *See* Neutral endopeptidase neprilysin (NEP)  
 Nephrocalcinosis, 592, 621  
 Nephrolithiasis, 404–405, 622  
 Nephrotic syndrome, 303–304, 638, 1261  
 Nephrotoxicity, 1119  
 NER. *See* Nucleotide excision repair (NER)  
 Nerve growth factor, 190  
 NETs. *See* Neutrophil extracellular traps (NETs)  
 Neurodegenerative diseases, 797  
 Neuropsychiatric outcomes, 217  
 Neuropsychiatric symptoms, 638  
 Neuropsychological functioning, 2  
 Neutral endopeptidase neprilysin (NEP), 286–287  
 Neutrophil extracellular traps (NETs), 1001  
 Neutrophil lymphocyte ratio (NLR), 1103  
 Neutrophils, 389, 745, 1001–1002
- New Dietary Strategies Addressing the Specific Needs of the Elderly Population for Healthy Aging in Europe study, The (NU-AGE study), 448  
 New vitamin D analogs evaluation with less calcemic activity, 1223–1224  
 New Western diet (NWD), 881–882  
 New Zealand, vitamin D status and prevalence of vitamin D deficiency in, 67t–68t  
 New Zealand-based ViDA trial, 831–832  
 Newborns, 316  
 Next-generation sequencing (NGS), 745  
 NF-kappa-B inhibitor alpha (NFKBIA), 924  
 NF-kB. *See* Nuclear factor kappa B (NF-kB)  
 NFAT. *See* Nuclear factor of activated T cells (NFAT)  
 NFATs. *See* Nuclear factors of activated T cells (NFATs)  
 NFKBIA. *See* NF-kappa-B inhibitor alpha (NFKBIA)  
 NFs. *See* Normal fibroblasts (NFs)  
 NGR. *See* Normal glucose regulation (NGR)  
 NGS. *See* Next-generation sequencing (NGS)  
 NHANES. *See* National Health and Nutrition Examination Survey (NHANES)  
 NHANES III. *See* National Health and Nutrition Examination Survey III (NHANES III)  
 NHB. *See* Non-Hispanic Black (NHB)  
 NHL. *See* Non-Hodgkin's lymphoma (NHL)  
 NHLBI. *See* National Heart, Lung, and Blood Institute (NHLBI)  
 NHPs. *See* Natural health products (NHPs)  
 NHS. *See* Nurses' Health Study (NHS)  
 NHW. *See* Non-Hispanic White (NHW)  
 Nicotinamide adenine dinucleotides (NAD), 982  
 NIH. *See* National Institutes of Health (NIH)  
 NIST. *See* National Institute of Standards and Technology (NIST)  
 Nitrate, 107–108  
 Nitric oxide (NO), 35, 107–108, 701, 969, 1000  
 pathway, 969  
 Nitroblue tetrazolium (NBT), 742  
 Nitrogen molecules, 971  
 Nitrogen oxides (NOX), 1048  
 Nitrogen species, 980  
 reduced generation of, 979–980  
 Nitrogen-containing BPs (N-BPs), 659  
 Nitrous anhydride, 969  
 Nivolumab, 903  
 NK cell large granular lymphocyte leukemia (NK-LGLL), 752  
 NK cells. *See* Natural killer cells (NK cells)

- NK-LGLL. *See* NK cell large granular lymphocyte leukemia (NK-LGLL)
- NKF. *See* National Kidney Foundation, The (NKF)
- NKT cells. *See* Natural killer T cells (NKT cells)
- NLR. *See* Neutrophil lymphocyte ratio (NLR)
- NLS. *See* Nuclear localization signal (NLS)
- NMF. *See* Nonnegative matrix factorization (NMF)
- NMSC. *See* Nonmelanomaskin cancer (NMSC)
- NNK. *See* 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)
- NNT. *See* Number needed to treat (NNT)
- NO. *See* Nitric oxide (NO)
- No observed adverse effect level (NOAEL), 21
- No-observable adverse-effect level (NOAEL), 128
- NOAEL. *See* No observed adverse effect level (NOAEL); No-observable adverse-effect level (NOAEL)
- Nocardia* infection, 670
- NOD. *See* Nucleotide oligomerization domain (NOD)
- NOD mouse. *See* Nonobese diabetic mouse (NOD mouse)
- NOD2 gene, 1000
- Non-Hispanic Black (NHB), 1168
- Non-Hispanic White (NHW), 1168
- Non-Hodgkin's lymphoma (NHL), 633
- Non-skeletal health in cats, role of vitamin D on, 670–671
- Non-skeletal health in dogs, role of vitamin D on, 667–670
- Nonalcoholic fatty liver disease (NAFLD), 501, 559, 813, 1003
- Nonalcoholic steatohepatitis (NASH), 559, 562, 813
- vitamin D/VDR signaling in, 562–563
- “Noncalcemic” VDR ligands, 562–563
- Nonclassical actions of vitamin D, 995
- Nonclassical immune cells, 1002
- Nonclassical pathways, 978
- Nondipper profile, 390
- Noninfectious etiologies, 632
- Nonmelanomaskin cancer (NMSC), 103
- Nonnegative matrix factorization (NMF), 951–952
- Nonnutritional rickets, radiological signs of, 232–235
- differential diagnosis of rickets from imaging perspective, 235–236
- HPP, 235
- hypophosphatemic rickets/osteomalacia, 233–234
- vitamin D-dependent rickets, 232–233
- Nonobese diabetic mouse (NOD mouse), 1112
- Nonrandom population sampling, 491
- Nonrenal 1 $\alpha$ -hydroxylase in CKD, 1,25(OH) $_2$ D corrects impaired 25(OH)D availability to, 596–599
- Nonsense mutations, 374–376
- Nonskeletal health outcomes, 663
- Non–small cell lung cancer (NSCLC), 901–902
- vitamin D and chemoprevention, 904–905
- Nonspecific esterase (NSE), 742
- Nonsteroidal analogs, 1118–1119
- Nonsteroidal antiinflammatory drugs (NSAIDs), 771
- Nonstructural 3 (NS3), 1011–1012
- Nonsurgical weight loss, comparison with, 163–164
- Nonsurgical weight loss, comparison with, 166
- Nontraditional risk markers, 574
- Nontransformed embryonic fibroblasts, 688–690
- Nontumorigenic mammary epithelial cells, enriched pathways in, 841–842
- Nonvitamin D benefits from solar UV exposure, 107–108
- 19-Nor-1 $\alpha$ , 959
- Nordic guidelines, 5
- Nordic Nutrition Recommendations, 5
- Normal calcium (NC), 636
- Normal fibroblasts (NFs), 878–879
- Normal glucose regulation (NGR), 542
- vitamin D and regression to, 543
- vitamin D supplementation and regression to, 542–543
- Normal healthy persons, 136
- Normal human mammary epithelial cells, 688
- Normal mammary gland, VDR expression and function in, 832–836
- Normal renal synthesis, 588–589
- Normal vitamin D homeostasis, 341
- North America
- current intakes of vitamin D in young and adult populations in, 138–143, 144t–145t
- vitamin D status in, 49
- NOX. *See* Nitrogen oxides (NOX)
- NPN. *See* Natural product number (NPN)
- NPT2a mutations, 282, 292
- NPT2b, 282
- NPT2c mutations, 282, 292–293
- NR41A. *See* Nuclear receptor subfamily 4 group A member 1 (NR41A)
- NRAS genes, 292
- NRF2. *See* Nuclear factor erythroid 2-related factor 2 (NRF2)
- Nrf2. *See* Nuclear factor-erythroid-2-related factor 2 (Nrf2)
- NRF2/antioxidant response element signaling pathway (NRF2/ARE signaling pathway), 771–772
- NRF2/ARE signaling pathway. *See* NRF2/antioxidant response element signaling pathway (NRF2/ARE signaling pathway)
- NS3. *See* Nonstructural 3 (NS3)
- NSAIDs. *See* Nonsteroidal antiinflammatory drugs (NSAIDs)
- NSCLC. *See* Non–small cell lung cancer (NSCLC)
- NSE. *See* Nonspecific esterase (NSE)
- NTCU. *See* N-nitroso-tris-chloroethylurea (NTCU)
- NU-AGE study. *See* New Dietary Strategies Addressing the Specific Needs of the Elderly Population for Healthy Aging in Europe study, The (NU-AGE study)
- Nuclear factor erythroid 2-related factor 2 (NRF2), 745–746
- Nuclear factor kappa B (NF-kB), 561–562, 799, 1092
- pathway, 805
- Nuclear factor of activated T cells (NFAT), 568–569
- Nuclear factor-erythroid-2-related factor 2 (Nrf2), 979
- Nuclear factors of activated T cells (NFATs), 1015–1016
- Nuclear localization signal (NLS), 374
- Nuclear receptor subfamily 4 group A member 1 (NR41A), 752–753
- Nuclear receptors, 560
- first description of genetic defect in nuclear receptor superfamily, 373–374
- Nucleocapsid (N), 1091
- Nucleotide excision repair (NER), 971
- Nucleotide oligomerization domain (NOD), 1058
- NOD1, 1058
- NOD2, 1058
- Nude mice, 476
- Number needed to treat (NNT), 422–423
- Nurses' Health Study (NHS), 516, 720, 1169
- and Diabetes Prevention Program cohorts, 525
- Nutrients, 11, 20, 162
- intake, 148, 154–155
- supplementation, 256
- threshold, 11
- Nutrition(al), 66
- clinical trials cannot match quality of evidence achieved with drugs, 15–17
- nutrition-related disorders, 17
- rickets, 242, 244–246, 248, 256
- epidemiology of, 242–246
- pathogenetic spectrum of, 265–268
- radiological features of, 231–232
- stages of calcium deprivation leading to, 230f
- UK scientific advisory committee on, 1–2
- vitamin D deficiency, 312–313
- NVD. *See* Vitamin D nanoemulsion (NVD)
- NWD. *See* New Western diet (NWD)

- O**
- 12-O-tetradecanoylphorbol-13-acetate (TPA), 745–746
- Obesity, 101, 161, 172, 492–493, 496–501, 503, 1042, 1060, 1132–1133
- bariatric surgery effective treatment for, 161–162
- dyslipidemia and nonalcoholic fatty disease of liver, 501
- and efficacy of oral vitamin D supplementation, 495–496
- inflammation and hormonal adipokines, 498–499
- insulin resistance, insulin secretion, and glycemia, 499–500
- vitamin D and hypertension, 500–501
- and vitamin D deficiency, 491–492
- Obesity Medicine Association, 175–176
- Observational cohort studies, 543–544
- Observational studies, 525–526, 827–832, 827t–828t, 1175–1177
- limitations of observational studies and issue of confounding, 526
- on vitamin D and type 2 diabetes, 515–526
- case–control and cross-sectional studies, 515–516
- limitations of observational studies and issue of confounding, 526
- longitudinal cohort studies, 516–525
- Mendelian randomization studies, 525–526
- seasonal and geographic studies, 515
- vitamin D status and cardiovascular outcomes in, 570–571
- Obstructive lung diseases, 1235
- OCA. *See* Ovarian Cancer Association (OCA)
- OCA2gene, 185–186
- Occludin, 1061
- Occupation, 103–104
- Oceania, vitamin D status in, 66
- OCLs. *See* Osteoclasts (OCLs)
- Ocular infection, 315–316
- Odds ratios (ORs), 123, 415
- ODIN project, 151–153
- ODS. *See* Office of Dietary Supplements (ODS)
- Office of Dietary Supplements (ODS), 130–131, 1271
- Ogg1. *See* 8-oxoguanine-DNA glycolase 1 (Ogg1)
- 1 $\alpha$ -OHase, 569
- deficiency of, 313–314
- Oily fish, 1061
- OLGs. *See* Oligodendrocytes (OLGs)
- Oligodendrocyte precursor cells (OPC), 1130–1131, 1150
- differentiation, 1152–1153
- Oligodendrocytes (OLGs), 1130, 1149–1155
- Omega-3 fatty acids, 34
- OMIM. *See* Online Mendelian Inheritance in Man compendium (OMIM)
- Onchorynchus* sp., 82
- Oncogene LIP, 600–601
- Oncogenes, 688–690
- Oncogenic osteomalacia, 293
- Oncology, 669
- One-anastomosis gastric bypass, 162
- Online Mendelian Inheritance in Man compendium (OMIM), 327, 401
- OPC. *See* Oligodendrocyte precursor cells (OPC)
- OPG. *See* Osteoprotegerin (OPG)
- Optimal vitamin D nutrition, 47
- OR. *See* Overall response (OR)
- Oral health, all-cause mortality, 2
- Oral microbiome, 938
- Oral pulse therapy, 629
- Oral vitamin D supplementation, obesity and efficacy of, 495–496
- ORC. *See* Origin recognition complex (ORC)
- Organ failure, severity of critical illness and, 1261
- Organ transplant(ation)
- effects of vitamin D on immunity and graft rejection, 475–476
- immune effects of vitamin D, 476
- role of vitamin D in allograft rejection, 475–476
- treatment of posttransplant bone loss with vitamin D and analogs, 480–482
- change in bone mineral density in renal transplant recipients treated with calcitriol or placebo, 480f
- vitamin D deficiency at time of, 478
- comparison of serum 25-hydroxyvitamin D levels, 479f
- vitamin D deficiency following, 478–480
- vitamin D deficiency at time of organ transplantation, 478
- vitamin D deficiency in long-term transplant recipients, 478–480
- vitamin D deficiency prior to, 476–478
- risk factors for vitamin D deficiency in organ transplant patients, 477t
- vitamin D deficiency in patients with chronic kidney disease, 478
- vitamin D deficiency in patients with congestive heart failure, 477
- vitamin D deficiency in patients with end-stage pulmonary disease, 476–477
- vitamin D deficiency in patients with liver failure, 477
- Organic form, 281
- Organic solvents, 1265–1266
- Organic solvents, antirachitic compounds
- vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in crystal form and dissolved in, 78–79
- Organoids, 880
- Organs, 86
- Origin recognition complex (ORC), 757–758
- ORs. *See* Odds ratios (ORs)
- Orthotopic allograft PDCA model, 812
- OS. *See* Overall survival (OS)
- OS/BS. *See* Osteoid surface relative to bone surface (OS/BS)
- Osimertinib (OSM), 902, 907
- OSM. *See* Osimertinib (OSM)
- Ossification process, 602
- Osteitis fibrosa cystica, 310–311
- Osteoblasts and osteocytes (OSVDR), 413
- Osteocalcin, 250
- Osteoclastogenesis, 304
- Osteoclasts (OCLs), 651
- express TAF12, transgenic mice with tartrate-resistance acid phosphatase–driven measles virus nucleocapsid gene expression in, 653
- formation in marrow cultures from PD patients expressing MVNP in OCL precursors, effects of transduction of TAF12 antisense construct on, 655
- mechanism of action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoclast formation, 652
- precursors, effects of IL-6 on TAF12 expression by, 654
- precursors, TAF12 binds Cyp24A1 promoter in, 655
- precursors from Paget's disease patients contribute to hyperresponsivity to 1,25(OH)<sub>2</sub>D<sub>3</sub>, increased levels of TAF12 in, 652–655
- precursors from Paget's patients, mechanism of action of TAF12 in increased 1,25(OH)<sub>2</sub>D<sub>3</sub> responsivity of, 655–656
- precursors from patients with Paget's disease reduced expression of TAF12, transduction of antisense measles virus nucleocapsid protein construct into, 654
- precursors from TRACP–TAF12 transgenic mice hyperresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub>, 655
- precursors induces TAF12, measles virus infection of, 653
- Osteocytes, 1262
- Osteogenesis, 246–247
- Osteoid surface relative to bone surface (OS/BS), 228
- Osteoid volume relative to bone volume (OV/BV), 228
- Osteomalacia, 223, 227–229, 233–234, 237, 281, 306, 327, 570, 625–626
- bone histomorphometry in, 227–228
- bone mineral density distribution in, 227–228
- development of, 306f
- imaging choice in, 236–237
- radiological features of, 229–232
- radiological signs of, 232–235
- stages of calcium deprivation leading to, 230f
- Osteonecrosis, 667
- Osteopenia, 252

- Osteopetro rickets, 235  
 Osteopontin, 262–263, 287  
 Osteoporosis, 306, 411–412, 443, 476–477, 480  
   determining optimal serum 25-hydroxyvitamin D concentrations for musculoskeletal health, 415–416  
   effects of active vitamin D analogs on fractures, 427  
   anabolic vitamin D analogs, 2MD, 427  
   vitamin D analogs, eldecalcitol, 427  
   effects of vitamin D alone or calcium and vitamin D on bone mineral density and falls, 416–418  
   effects of vitamin D on skeleton, 412–413  
   large randomized controlled trials of vitamin D supplementation, 427–428  
   management of osteoporosis after bariatric surgery, 177  
   mendelian randomization studies, 428  
   metaanalyses, 423–425  
     calcium plus vitamin D supplementation *vs.* placebo and total fracture, 424f  
   primary fracture prevention with vitamin D or calcium and vitamin D, 418–423  
     cumulative incident total, nonvertebral, and hip fractures, 421f  
     primary fracture prevention with calcium and vitamin D, 422–423  
     single high annual or monthly doses of vitamin, 420–422  
     effect of vitamin D alone on fractures, 418–420  
   role of vitamin D genetic factors in osteoporosis and possible interactions with vitamin D therapy, 413–414  
   safety, 425–427  
     calcium and vitamin D, 425–426  
     secondary fracture prevention with vitamin D or calcium and vitamin D, 426  
     vitamin D, 425  
   seasonal variation in vitamin D status, 414–415  
 Osteoporosis Risk Factor and Prevention (OSTPRE), 451  
 Osteoprotegerin (OPG), 304, 412, 602, 652  
 Osteosarcoma cells, 635  
 OSTPRE. *See* Osteoporosis Risk Factor and Prevention (OSTPRE)  
 OSVDR. *See* Osteoblasts and osteocytes (OSVDR)  
 OSVDR mice, 413  
 OTC. *See* Over the counter (OTC); Over-the-counter (OTC)  
 Outdoor  
   recreational activities, 104  
   workers, 103–104  
 OV/BV. *See* Osteoid volume relative to bone volume (OV/BV)  
 Ovarian cancer, 682  
 Ovarian Cancer Association (OCA), 214  
 Over the counter (OTC), 126–127  
 Over-irradiation products, 972–973  
 Over-the-counter (OTC), 125  
 Overall response (OR), 1014–1015  
 Overall survival (OS), 864, 958  
 Oxacalcitriol, 630  
 22-oxacalcitriol, 630  
 Oxidants, 80–81  
 Oxidative DNA damage, 885  
 Oxidative phosphorylation (OxPhos), 1044  
 Oxidative stress, 476  
 8-oxoguanine-DNA glycolase 1 (Ogg1), 971–972  
 OxPhos. *See* Oxidative phosphorylation (OxPhos)  
 P  
 P-glycoprotein, 700  
 p. K242N mutation, 345  
 p. L99P mutation, 345, 353  
 4p12 polymorphisms, 413–414  
 P1NP. *See* Procollagen type I intact N-terminal propeptide (P1NP); Propeptide of type 1 procollagen (P1NP)  
 p38, 799  
 p53 tumor suppressor gene, 755, 980–981  
 P62<sup>P392L</sup> Paget's patients hyperresponsive to 1,25(OH)<sub>2</sub>D<sub>3</sub> and display enhanced RANKL production in response to 1,25(OH)<sub>2</sub>D<sub>3</sub>, 657  
 p62<sup>P394L</sup> knock-in mice (KI mice), 657  
   marrow stromal cells derived from, 657  
 PADI1. *See* Peptidyl arginine deiminase 1 (PADI1)  
 Paget's disease (PD), 651  
   increased levels of TAF12 in osteoclast precursors from Paget's disease patients contribute to hyperresponsivity to 1,25(OH)<sub>2</sub>D<sub>3</sub>, 652–655  
   effects of IL-6 on TAF12 expression by osteoclast precursors, 654  
   measles virus infection of osteoclast precursors induces TAF12, 653  
   overexpression of TAF12 sufficient to induce 1,25(OH)<sub>2</sub>D<sub>3</sub> hyperresponsivity in transduction of antisense measles virus nucleocapsid protein construct into osteoclast precursors from patients with Paget's disease  
   reduced expression of TAF12, 654  
   transgenic mice with tartrate-resistance acid phosphatase-driven measles virus nucleocapsid gene expression in OCLs express TAF12, 653  
   mechanism of action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoclast formation, 652  
   patients contribute to hyperresponsivity to 1,25(OH)<sub>2</sub>D<sub>3</sub>, increased levels of TAF12 in osteoclast precursors from, 652–655  
   reduced expression of TAF12, transduction of antisense measles virus nucleocapsid protein construct into osteoclast precursors from patients with, 654  
 Paget's patients  
   mechanism of action of TAF12 in increased 1,25(OH)<sub>2</sub>D<sub>3</sub> responsivity of OCL precursors from, 655–656  
   serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in, 659  
 Pale skin, 105  
 Pamidronate, 405  
 PAMPs. *See* Pathogen-associated molecular patterns (PAMPs)  
 Pancreatic beta-cell function, 512–513  
 Pancreatic cancer (PC), 811–812, 937–939, 939f  
   biology and genetics of, 950–954  
     pancreatic cancer molecular subtypes, 951–952  
     somatic genetics of, 950–951  
     tumor microenvironment in, 952–954  
   biology of vitamin D in pancreatic cancer, 954–957  
     vitamin D and pancreatic tumor cells, 954–956  
     vitamin D and pancreatic tumor microenvironment, 956–957  
   clinical presentation and treatment, 938–939  
   epidemiological studies, 939–950, 940t–943t  
   ethnicity, 948  
   etiology of, 937–938  
   genetic susceptibility, 938  
   genetics, 949  
   serum/plasma levels of vitamin D, 949–950  
     circulating vitamin D-binding protein, 950  
     predicted levels of plasma 25(OH)D, 950  
   vitamin D insufficiency/deficiency in patients with PC, 950  
   vitamin D status, 950  
   solar ultraviolet-B radiation, 939–947  
   studies reporting on, 944t  
   vitamin D and prognosis in patients with PC, 957–958  
   vitamin D intervention studies in patients with, 945t–946t, 958–959  
 Pancreatic diseases, 668  
 Pancreatic ductal adenocarcinoma (PDAC), 811, 937  
 Pancreatic insufficiency, 313  
 Pancreatic Intraepithelial Neoplasms (PanIN), 950–951  
 Pancreatic-stellate cells (PSCs), 811, 952  
 PanIN. *See* Pancreatic Intraepithelial Neoplasms (PanIN)



- Papilledema, 306
- PAR. *See* Population attributable risk (PAR)
- Paracalcitol, 695
- Paracoccidioidomycosis, 632
- Paracrine actions in CKD, 596–599
- Parainfluenza, 1014
- Parathormone, 216, 401
- Parathyroid glands, 310, 595–596
- damage/destruction, 310
- disorders of parathyroid gland formation, 306–309
- Parathyroid hormone (PTH), 99, 126, 167, 229–231, 249, 263, 281–282, 303, 341, 361, 459, 513–514, 568–569, 574–577, 587, 592, 625–626, 664, 687–688, 1047, 1260
- in acute defense of serum calcium concentration, 304
- availability, hypocalcemia due to abnormalities of, 306, 307t–309t
- disorders of parathyroid hormone production or secretion, 309–310
- hypocalcemia due to resistance to actions of, 310–311
- parathyroid hormone-independent hypocalcemia, 312–313
- neonatal hypocalcemia, 312
- nutritional vitamin D deficiency, 312–313
- vitamin D malabsorption, 313
- replacement therapy, 317–318
- suppression of parathyroid hormone secretion, 461–462
- Parathyroid hormone-related peptide (PTHrP), 224, 263, 633, 687–688
- Parathyroid hyperplasia and 1,25(OH)<sub>2</sub>D resistance in CKD, pathogenesis of, 600f
- Paraventricular nucleus (PVN), 515
- Parent compound, 634–635
- Parent vitamin D to restore vitamin D health, advantages and disadvantages of, 460
- Paricalcitol, 630, 769, 774, 959
- Parkinson's disease, 217
- PARPs. *See* Poly(ADP-ribose) polymerases (PARPs)
- Particle disease, 632
- PASI. *See* Psoriasis area and severity index (PASI)
- Patched signaling (Ptch signaling), 973–974
- Pathogen recognition receptors (PRRs), 1035
- Pathogen-associated molecular patterns (PAMPs), 460, 1012, 1035, 1185–1186
- Pathogenetic spectrum of nutritional rickets, 265–268
- Pathogenic wound healing process, 562
- Pathophysiology of secondary hyperparathyroidism (SHPT), 462
- Pattern recognition receptors (PRRs), 995, 1012, 1058, 1185–1186
- 1,25(OH)<sub>2</sub>D promotes expression of, 999–1000
- PBMCs. *See* Peripheral blood mononuclear cells (PBMCs)
- PC. *See* Pancreatic cancer (PC)
- PCa. *See* Prostate cancer (PCa)
- PCNA. *See* Proliferating cell nuclear antigen (PCNA)
- PCR. *See* Polymerase chain reaction (PCR)
- PCSK9 gene, 187–189
- PD. *See* Paget's disease (PD)
- PD-1. *See* Programmed cell death protein 1 (PD-1)
- PD-L1. *See* Programmed death ligand 1 (PD-L1)
- PDAC. *See* Pancreatic ductal adenocarcinoma (PDAC)
- pDCs. *See* Plasmacytoid DCs (pDCs)
- PDDR. *See* Pseudo-vitamin D deficiency rickets (PDDR)
- PDDR-II. *See* Pseudovitamin D deficiency rickets type II (PDDR-II)
- PDGFRβ, 562–563
- PDIA3. *See* Protein disulfide isomerase family A member 3 (PDIA3)
- PDIA7, 192
- PDILTd. *See* Protein disulfide isomerase like, testis expressed (PDILTd)
- Pediatric onset MS, 1178–1179
- PEDV. *See* Porcine epidemic diarrhea virus (PEDV)
- Pembrolizumab, 903
- Pemphigus foliaceus (PF), 1218
- Pemphigus vulgaris (PV), 1218
- Peptide hormones, 459
- Peptide YY, 172–173
- Peptidyl arginine deiminase 1 (PADI1), 189
- Per-protocol, 467
- Peripheral blood mononuclear cells (PBMCs), 596, 1038, 1187–1188, 1212, 1264
- Peripheral insulin, 513–514
- Peripheral mononuclear cells, 372–373
- Peripheral nervous system, 284
- Peripheral quantitative computed tomography (pQCT), 233, 448
- Peripheral vascular disease, 511–512
- Pernicious anemia, 310
- Peroxisomal Biogenesis Factor 10 (PEX10), 189
- Peroxisome proliferator-activated receptor-γ (PPARγ), 560
- Peroxisome proliferator-activated receptor delta (PPAR-d), 513–514
- Peroxynitrite, 969
- Persistent Müllerian Duct Syndrome (PMDS), 378
- Personalized medicine, 902–903
- Petrolatum, 1215
- PEX10. *See* Peroxisomal Biogenesis Factor 10 (PEX10)
- PF. *See* Pemphigus foliaceus (PF)
- PFDN4. *See* Prefoldin subunit 4 (PFDN4)
- PFKFB4. *See* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 (PFKFB4)
- PFS. *See* Progression-free survival (PFS)
- PG. *See* Prostaglandin (PG)
- PGE<sub>2</sub>. *See* Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)
- PHA. *See* Phytohemagglutinin-A (PHA)
- Phagocytosis, 1076
- Phagosomes, 1076
- Pharmaceuticals, 17
- Pharmacokinetics, 699
- Phe251Cys. *See* Phenylalanine to cysteine at amino acid 251 (Phe251Cys)
- Phenobarbital, 315–316
- Phenotype, 836
- Phenotypic malleability, 1138
- Phenylalanine to cysteine at amino acid 251 (Phe251Cys), 379
- Phenytoin, 1261
- PHEX gene, 233
- Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), 745–746
- Phosphatase and tensin homolog (PTEN), 981
- Phosphate (PO<sub>4</sub>), 225–226, 263–264, 281, 363–364
- balance, 282–283
- binders, 317
- deprivation, 294–295
- homeostasis, 281–284, 995
- acquired hypophosphatemic disorders with elevated FGF23 levels, 293
- biochemical findings in, 287t
- clinical symptoms of hypophosphatemia and hyperphosphatemia, 284
- dietary phosphate regulates NPT2a and NPT2c, 283f
- disorders of, 284–295, 285t
- disorders of altered phosphate load, 294–295
- Fanconi's syndrome, 284–285
- genetic hyperphosphosphatemic disorders, 293–294
- genetic hypophosphatemic disorders with low FGF23 levels, 292–293
- genetic hypophosphatemic syndromes of excess FGF23, 285–292
- molecular pathways underlying genetic disorders of, 286f
- phosphate and vitamin D metabolism, 284
- regulation of, 282–284
- phosphate-regulated apoptosis, 225
- phosphate-restrictive diets, 317
- restriction, 284
- sensor, 599–600
- supplementation, 265
- treatment and supplementation of, 264t
- Phosphatidylinositol-3,4,5-trisphosphate (PIP3), 981
- Phosphatidylinositol-4,5-bisphosphate (PIP2), 981

- Phosphatonin, 282–283  
 Phosphaturia, 229–231  
 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4), 1117–1118  
 Phosphoinositide 3-kinase (PI3-K), 747–749, 981  
 Phosphoinositide 3-kinase/AKT pathway, 747–749  
 Phospholipase A2 group III (PLA2G3), 192  
 Phosphopenic, phosphate homeostasis, 225  
 Phosphorus, 253, 263, 412  
 Phosphorus excretion, 243  
 Photoallergenicity testing methodology, 1215  
 Photocarcinogenesis, vitamin D system and, 973–974  
 Photoimmune suppression, 972  
 6–4photoproducts, 968–969  
 Photoprotection, 975  
 Photoreactivation, 971  
 PHP. *See* Pseudo-hypoparathyroidism (PHP)  
 PHPT. *See* Primary hyperparathyroidism (PHPT)  
 Phytochemicals, 771–772  
 Phytohemagglutinin-A (PHA), 372–373, 767  
 Pi. *See* Inorganic phosphate (Pi); Inorganic phosphorus (Pi)  
 PI3-K. *See* Phosphoinositide 3-kinase (PI3-K)  
 PIA. *See* Proliferative inflammatory atrophy (PIA)  
 Pigs, 85–86  
 PIN. *See* Precursors of intraepithelial neoplasias (PIN)  
 PIP2. *See* Phosphatidylinositol-4,5-bisphosphate (PIP2)  
 PIP3. *See* Phosphatidylinositol-3,4,5-trisphosphate (PIP3)  
 PKA. *See* Activate protein kinase A (PKA); Protein kinase A (PKA)  
 PKC. *See* Protein kinase C (PKC)  
 PLA2G3. *See* Phospholipase A2 group III (PLA2G3)  
 PLAB. *See* Prostate differentiation factor (PLAB)  
 Placebo on areal bone mineral density in general population, 447–448  
 Placebo on fractures in high-risk populations, RCTs of supplemental vitamin D *vs.*, 450–451  
 Placebo on incident fractures in general population, RCTs of supplemental vitamin D *vs.*, 448–450  
 Placebo on volumetric bone mineral density, RCTs of supplemental vitamin D *vs.*, 448  
 Placebo-controlled trial, 41  
 Plant milks, 147–148  
 Plants, vitamin D in food farmed by, 84–87  
 Plaque psoriasis, 1208  
 Plasma, 281  
 Plasma 25(OH)D, predicted levels of, 950  
 Plasma levels of vitamin D, 949–950  
 Plasmacytoid DCs (pDCs), 1212  
 Platelets, 745  
 PLCG1 gene, 1148  
 PLE. *See* Protein losing enteropathy (PLE)  
 PLK1. *See* Polo-like kinase-1 (PLK1)  
 PLN. *See* Protein losing nephropathy (PLN)  
 PLZF. *See* Promyelocytic leukemia zinc finger (PLZF)  
 PMDS. *See* Persistent Müllerian Duct Syndrome (PMDS)  
 PML/RAR $\alpha$ . *See* Promyelocytic leukemia-retinoic acid receptor alpha (PML/RAR $\alpha$ )  
*Pneumocystis jirovecii* pneumonia, 632  
 Policy-makers, 16–17  
 Polo-like kinase-1 (PLK1), 758  
 Poly(ADP-ribose) polymerases (PARPs), 982  
 Polycystic kidney disease, 602–603  
 Polydipsia, 638, 667  
 Polymerase chain reaction (PCR), 373, 1100–1103  
 Polymers, 562–563  
 Polymorphisms, 1241–1242  
   in CYP24A1 gene and cardiovascular outcomes, 574  
   in VDR and vitamin D-binding protein genes, effects of, 1100  
   in VDR gene and cardiovascular outcomes, 574  
 Polyoma Virus middle T antigen in the mammary gland (MMTV-PyMT mice), 840–841  
 Polypeptide N-acetylgalactosaminyl-transferase 3 (GALNT3), 294  
 Polyploidization, 763  
 Polyuria, 667  
 Population attributable risk (PAR), 938  
 Population health, 2–3  
 Population studies, 31, 476, 827  
 Porcine epidemic diarrhea virus (PEDV), 1014  
 Pork, 85–86  
*Porphyromonas gingivalis*, 938  
 post-RC. *See* Postreplicative complex (post-RC)  
 Postreplicative complex (post-RC), 757–758  
 Posttransplant bone loss with vitamin D and analogs, treatment of, 480–482  
 Potential mechanisms, 1250  
   atopy and allergies, 1250  
   immunity, 1250  
 Poverty, 123  
 PPAR-d. *See* Peroxisome proliferator-activated receptor delta (PPAR-d)  
 PPAR $\gamma$ . *See* Peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ )  
 PPHP. *See* Pseudopseudohypoparathyroidism (PPHP)  
 PPI. *See* Inorganic pyrophosphate (PPI); Intopyrophosphate (PPI)  
 PPIs. *See* Prophylactic proton pump inhibitors (PPIs)  
 pQCT. *See* Peripheral quantitative computed tomography (pQCT)  
 PR. *See* Progesterone receptor (PR)  
 pre-RC. *See* Prereplicative complex (pre-RC)  
 Prebiotics, 876–877  
 Preclinical models of prostate, effects of vitamin D and analogs in, 920–923  
 Precursors of intraepithelial neoplasias (PIN), 808  
 Prediabetes, vitamin D supplementation for prevention of type 2 diabetes in people with, 533–543  
 Predicted 25(OH)D levels, measures of, 35–36  
 PREDIMED. *See* PREvencion con Dieta MEDiterranea (PREDIMED)  
 Preeclampsia, 404  
 Prefoldin subunit 4 (PFDN4), 192  
 Pregnancy, 404, 639, 1143  
   pregnancy-related outcomes, 217  
   studies suggest hormonally triggered reversible mechanism supporting reproductive success triggers MS remissions, 1136–1137  
 Pregnane X receptor (PXR), 560, 1261  
 “Prehypertrophic” chondrocytes, 224  
 Premature ovarian failure, 310  
 Prematurity of prematurity, rickets of, 263–265  
 Prenatal diagnosis, 385  
 Prereplicative complex (pre-RC), 757–758  
 Pretransplant vitamin D insufficiency, 475–476  
 PREvencion con Dieta MEDiterranea (PREDIMED), 1060  
 Previtamin D<sub>3</sub>, 1260  
 PRI2205, 909  
 Primary disease prevention, 16  
 Primary fracture prevention with calcium and vitamin D, 418–423  
 Primary hyperparathyroidism (PHPT), 625, 627, 639  
   PHPT-administered calcifediol, 628  
 Primary prevention, 16  
 Primary progressive disease course, 1167–1168  
 Primary spongiosa, 224  
 Primate biology, 15  
 Pro-apoptotic genes, 980  
 Probiotics, 1060  
 Procollagen type I intact N-terminal propeptide (P1NP), 417  
 Prodifferentiating, 1222  
 Progesterone, 373  
 Progesterone receptor (PR), 825–826  
 Programmed cell death protein 1 (PD-1), 908, 954

- Programmed death ligand 1 (PD-L1), 801–802, 908
- Progression-free survival (PFS), 864
- Prohormone, 687
- Proinflammatory  
actions of vitamin D on  
proinflammatory T lymphocyte  
responses, 1037  
cytokines, 498, 561–562  
tumor necrosis factor- $\alpha$ , 575
- Project Viva, 1236
- Prolactin, 387
- Proliferating cell nuclear antigen (PCNA), 759–760
- Proliferation, 691
- Proliferative inflammatory atrophy (PIA), 805, 808
- Promyelocytic leukemia zinc finger (PLZF), 691, 746
- Promyelocytic leukemia-retinoic acid receptor alpha (PML/RAR $\alpha$ ), 746
- Propeptide of type 1 procollagen (P1NP), 216
- Prophylactic proton pump inhibitors (PPIs), 170
- Prostaglandin (PG), 874–875
- Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), 799
- Prostaglandin pathway, 805
- Prostaglandin-endoperoxide synthase (PTGS-2), 874–875
- Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial, 722
- Prostate cancer (PCa), 36–37, 722, 726–727, 808–809, 917–918  
epidemiologic associations between PCa and vitamin D metabolites, 918–920  
25-Hydroxyvitamin D status and disparity of PCa in men of African genomic ancestry, 920  
heterogenous disease, 917–918  
high-dimensional data approaches to understanding VDR functions in prostate, 923–925  
lessons from PCa clinical trials with vitamin D compounds, 925–927  
chemoprevention of PCa by vitamin D supplementation, 925–926  
chemotherapy of PCa with 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs and impact of genomic ancestry, 926–927  
rationale to treat PCa by targeting VDR, 920–923  
effects of vitamin D and analogs in preclinical models of prostate, 920–923
- Prostate differentiation factor (PLAB), 921
- Prostate-specific antigen (PSA), 683–684, 722, 918
- Protease inhibitors, 846
- Proteasomal degradation, 861
- Protein disulfide isomerase family A member 3 (PDIA3), 680, 749–750
- Protein disulfide isomerase like, testis expressed (PDILTd), 192
- Protein kinase A (PKA), 304
- Protein kinase C (PKC), 282, 304  
activators, 771  
signaling, 746–747
- Protein losing enteropathy (PLE), 666–667
- Protein losing nephropathy (PLN), 670
- Protein Tyr kinase 7 (PTK7), 751
- Proteinuria, 479
- Proteomic approach, 1243
- Proto-oncogenes, 688–692
- Proton pump inhibitors, 168
- Provitamin D, 78
- PRRs. *See* Pathogen recognition receptors (PRRs); Pattern recognition receptors (PRRs)
- PRSS1 mutation, 938
- PRSS2 mutation, 938
- PSA. *See* Prostate-specific antigen (PSA)
- PSCs. *See* Pancreatic-stellate cells (PSCs)
- Pseudo-hypoparathyroidism (PHP), 310–311, 318, 625–626
- Pseudo-vitamin D deficiency rickets (PDDR), 327
- Pseudomonas aeruginosa*, 997–998, 1017, 1263–1264
- Pseudopseudohypoparathyroidism (PPHP), 311
- Pseudovitamin D deficiency rickets type II (PDDR-II), 359–360
- Psoralen plus UV-A (PUVA), 1216–1217
- Psoriasis, 1049, 1207  
biological effects of vitamin D and analogs, 1211–1214  
clinical use of 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs, 1214–1217  
histological demonstration of morphological changes in lesional psoriatic skin, 1209f  
pathogenesis, 1208  
vitamin D system in normal and psoriatic skin, 1208–1211
- Psoriasis area and severity index (PASI), 1212
- Psoriatic lesions treatment in HIV patients, 1216
- Psoriatic skin, 1208
- Ptch signaling. *See* Patched signaling (Ptch signaling)
- PTEN. *See* Phosphatase and tensin homolog (PTEN)
- Pten mutation, 922
- PTGS-2. *See* Prostaglandin-endoperoxide synthase (PTGS-2)
- PTH. *See* Parathyroid hormone (PTH)
- PTHr1. *See* PTHrP receptors (PTHr1)
- PTHrP. *See* Parathyroid hormone–related peptide (PTHrP)
- PTHrP receptors (PTHr1), 282
- PTK7. *See* Protein Tyr kinase 7 (PTK7)
- Puberty, 385–387
- Public health measures, safety considerations and prerequisite data required prior to initiating, 154–155
- Public health policy, 115–116
- Pulmonary consumption, 1079
- Pulmonary diseases, 1014
- Pulmonary vascular permeability index (PVPI), 1269
- Pulse wave velocity (PWV), 575–576
- PUVA. *See* Psoralen plus UV-A (PUVA)
- PV. *See* Pemphigus vulgaris (PV)
- PVN. *See* Paraventricular nucleus (PVN)
- PVPI. *See* Pulmonary vascular permeability index (PVPI)
- PWV. *See* Pulse wave velocity (PWV)
- PXR. *See* Pregnane X receptor (PXR)
- Pyrimidine dimers, 101, 972
- ## Q
- 22q11.2 deletion syndrome (22q11.2DS), 306–309
- qBEI. *See* Quantitative backscattered electron imaging (qBEI)
- QCT. *See* Quantitative computed tomography (QCT)
- qPCR arrays, 691
- qRT-PCR. *See* Quantitative reverse transcription polymerase chain reaction (qRT-PCR)
- QT interval corrected for heart rate (QT<sub>C</sub>), 306
- Quality of life, 1057–1058
- Quantitative backscattered electron imaging (qBEI), 228–229
- Quantitative computed tomography (QCT), 164, 229
- Quantitative reverse transcription polymerase chain reaction (qRT-PCR), 1214
- ## R
- R-Spondin (RSPO), 861
- R-spondin family of proteins (RSPO1–4), 861
- R-square, 35
- RA. *See* Retinoic acid (RA); Rheumatoid arthritis (RA)
- RAAS. *See* Renin–angiotensin–aldosterone system (RAAS)
- Rachitic rosary, 246, 328–329
- Radiograph, 252
- Radiographic global impression of change (RGI-C), 251–252
- Radiographic severity score (RSS), 251–252
- Radiographs, 229–231, 256
- Radiolabeled 1,25(OH)<sub>2</sub>D<sub>3</sub> binding detection, 1149
- Radiological severity score (RSS), 231–232
- Rag. *See* Recombination activating gene (Rag)
- Ral-binding protein 1-associated Eps domain-containing 2 (REPS2), 869
- Randomized controlled interventions, 728–729

- Randomized controlled trials (RCTs), 31, 41–42, 104–105, 137–138, 145–148, 175–176, 201–202, 411–412, 415, 438, 443, 461, 498, 568, 723, 728–730, 831, 926, 1003–1004, 1082, 1094, 1177, 1231
- clinical and public health importance, 443
- large randomized controlled trials of supplemental vitamin D on fractures, 444t–445t
- of effects of vitamin D supplementation on bone density, 447–448
- of supplemental vitamin D *vs.* placebo on areal bone mineral density in findings from systematic reviews and metaanalyses, 443–446
- systematic reviews and metaanalyses of supplemental vitamin D and calcium on incident fractures, 446
- systematic reviews and metaanalyses of supplemental vitamin D on incident fractures, 443–446
- recommended intakes based on bone health goals, 446–447
- studies of vitamin D and total cancer incidence, 723–724
- Randomized Evaluation of Calcium Or vitamin D (RECORD), 532
- Randomized placebo controlled trial, 479
- RANK. *See* Receptor activator of nuclear factor kappa-B (RANK)
- RANKL. *See* Receptor activator of nuclear factor kappa-B ligand (RANKL)
- Rapamycin, 1119
- Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR), 749
- RAR. *See* Retinoic acid receptor (RAR)
- Rare inherited disorders, 284–285
- RAS. *See* Renine angiotensin system (RAS)
- RAS-transformed MCF10A (MCF10A-ras), 846–847
- Rat CKD models, 602
- Rayleigh scattering varies, 102
- Rayleigh's model, 100
- RBP receptor, 562–563
- RCTs. *See* Randomized controlled trials (RCTs)
- RDA. *See* Recommended dietary intake (RDA)
- RDAs. *See* Recommended Dietary Allowances (RDAs)
- Reactive oxygen, 980
- reduced generation of, 979–980
- Reactive oxygen species (ROS), 562–563, 999, 1092, 1151–1152, 1219, 1264
- 1,25(OH)<sub>2</sub>D regulates release of, 1000
- Receptor activator of nuclear factor kappa-B (RANK), 304
- Receptor activator of nuclear factor kappa-B ligand (RANKL), 172
- Receptor Tyr kinase-like orphan receptor family (ROR1 and ROR2), 751
- Recombination activating gene (Rag), 1065
- Recommended Dietary Allowances (RDAs), 3, 117, 242–243, 446, 494, 627
- Recommended dietary intake (RDA), 16
- Recurrence-free survival (RFS), 905
- Reference method procedures (RMPs), 666
- Reference nutrient intake (RNI), 2
- Reflectometry, 35
- Regression to normal glucose regulation, vitamin D and, 543
- Regression to normal glucose regulation, vitamin D supplementation and, 542–543
- Regulation of breast cancer stem cell populations, 847–848
- Regulation of cell cycle progression, 756–758
- control of S phase and DNA replication licensing, 757–758, 757f
- G2 and M phase transition, 758
- progression through G1 and G1/S boundary, 756–757
- Regulatory B cells (Breg), 1186
- Regulatory T cells (Treg), 798–799, 1019, 1060, 1186–1188
- Regulatory T lymphocytes, 1041
- actions of vitamin D on, 1041–1043
- Reinoid X receptor (RXR), 664–665
- Relapse rates, 1177
- Relapsing-remitting multiple sclerosis (RRMS), 1130, 1167–1168
- Related hospitalizations, 1014
- Relative risk (RR), 17, 415, 720
- RelB promoters, 380
- Remdesivir, 1095–1099
- Renal 1,25(OH)<sub>2</sub>D production, 592
- Renal 1,25(OH)<sub>2</sub>D synthesis, alterations in, 592–594
- Renal 1 $\alpha$ -OHase in vitamin D toxicity, control of, 636
- Renal calcitriol
- production in mineral and skeletal homeostasis, 588f
- synthesis in CKD, 591f
- Renal calcium excretion, 401–402
- Renal cell carcinoma, 687
- Renal CYP27B1, 413
- Renal disease, 265
- alterations in renal 1,25(OH)<sub>2</sub>D synthesis and 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism in
- alterations in vitamin D bioactivation to 25(OH)D in chronic kidney disease, 589–592
- defective renal 25(OH)D uptake limits renal calcitriol synthesis in CKD, 591f
- systemic and parathyroid vitamin D metabolism and actions, 590f
- central role of renal calcitriol production in mineral and skeletal homeostasis, 588f
- renal regulation of vitamin D endocrine system, 588–589
- Renal dysplasia, 309
- Renal failure, 625–626
- Renal FGF23/klotho axis, vitamin D maintenance of, 603–606
- Renal function, 638
- Renal ischemia reperfusion (IRI), 1266–1267
- Renal klotho contributes to renal and cardiovascular protection, vitamin D maintenance of, 606f
- Renal klotho levels, vitamin D control of hypertension and systemic inflammation conferring renal and cardiovascular protection independently of, 606–609
- Renal reabsorption, 282
- Renal regulation of vitamin D endocrine system, 588–589
- Renal transplant recipients trial, 479
- Renal tubular reabsorption of phosphate, 264
- Renin inhibitors, 575
- Renin–angiotensin–aldosterone system (RAAS), 568–569, 575, 1267
- hypertension, 577
- interrelationships between RAAS, parathyroid hormone, and cardiovascular system, 576f
- Renine angiotensin system (RAS), 512, 1092
- Replacement therapy, 332
- Replication protein A (RPA), 971
- Reproductive health, 2
- REPS2. *See* Ral-binding protein 1-associated Eps domain-containing 2 (REPS2)
- “Rescue diet”, 569–570, 602
- Research units (RU), 568–569
- Residual confounding, 1084
- Resistance and vitamin D metabolism, 699–701
- Respiratory disorders, 1232–1235
- asthma, 1233–1234
- childhood wheezing, 1233
- chronic obstructive pulmonary disease, 1234–1235
- potential mechanisms, 1248
- respiratory infections, 1232–1233
- Respiratory infections, 69, 1232
- Respiratory muscle weakness, 284
- Respiratory syncytial virus (RSV), 1012, 1014, 1094, 1232
- Respiratory tract infections (RTIs), 2, 1014
- vitamin D and, 1094
- Respiratory viral infections, 1014
- Resting zone, 224
- Restriction enzyme, 1265
- Restriction fragment length polymorphism, 1174
- Retention of vitamin D, 87–88
- Retention studies, 87
- Retinoblastoma protein control of 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced G1 block, 762
- Retinoic acid (RA), 192, 1222
- Retinoic acid receptor (RAR), 374
- RAR $\alpha$ , 754



- Retinoic acid receptor (RAR) (*Continued*)  
 RAR $\beta$ , 770–771  
 Retinoic acid–inducible gene 1 (RIG-I), 1016–1017  
 Retinoic orphan receptor  $\gamma$  (ROR $\gamma$ ), 1218  
 Retinoic acid–inducible gene 1 (RIG-I), 1016–1017  
 Retinoid X receptor (RXR), 364, 596–597, 1015–1016, 1209–1210  
 RXR $\alpha$ , 742, 1209–1210  
 Retinoids, 770–771  
 Reverse causation, 40  
 RFS. *See* Recurrence-free survival (RFS)  
 RGI-C. *See* Radiographic global impression of change (RGI-C)  
 Rhabdomyolysis-induced acute renal failure, 315  
 Rheumatoid arthritis (RA), 2, 1058, 1185  
 Rhinovirus, 1014  
 Rho GTPases, 1212  
 Riboflavin, 80–81  
 Ribonucleic acid (RNA), 1091  
 Ribosomal S6 kinase, 764–765  
 Rickets, 223, 235, 241–242, 252, 306, 313, 327, 345, 352, 570. *See also* Vitamin  
 Rickets, radiological features of, 229–231  
 RICTOR. *See* Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR)  
 Rifampicin, 1075  
 Rifampin, 405–407, 621  
 RIG-I. *See* Retinoic acid–inducible gene 1 (RIG-I); Retinoic acid–inducible gene 1 (RIG-I)  
 RIG-I-like receptors (RLR), 1016–1017  
 Ring finger protein 43 (RNF43), 861  
 Risk groups in worldwide vitamin D status, 66  
 RLR. *See* RIG-I-like receptors (RLR)  
 RMPs. *See* Reference method procedures (RMPs)  
 RNA. *See* Ribonucleic acid (RNA)  
 RNA-seq, 880  
 RNF43. *See* Ring finger protein 43 (RNF43)  
 RNI. *See* Reference nutrient intake (RNI)  
 Rodent EAE, 1139  
 Rodent models, 569  
 ROR1 and ROR2. *See* Receptor Tyr kinase-like orphan receptor family (ROR1 and ROR2)  
 ROR $\gamma$ . *See* Retinoic orphan receptor  $\gamma$  (ROR $\gamma$ )  
 ROS. *See* Reactive oxygen species (ROS)  
 Rosai-Dorfman disease, 632–633  
 Rotavirus, 1014  
 Roux limb, 162  
 Roux-en-Y gastric bypass (RYGB), 162, 164, 295  
 RPA. *See* Replication protein A (RPA)  
 RR. *See* Relative risk (RR)  
 RRMS. *See* Relapsing-remitting multiple sclerosis (RRMS)  
 RSPO. *See* R-Spondin (RSPO)  
 RSPO1–4. *See* R-spondin family of proteins (RSPO1–4)  
 RSS. *See* Radiographic severity score (RSS); Radiological severity score (RSS)  
 RSV. *See* Respiratory syncytial virus (RSV)  
 RTIs. *See* Respiratory tract infections (RTIs)  
 RU. *See* Research units (RU)  
 Runt-related transcription factor 1 (RUNX1), 752  
 RUNX1. *See* Runt-related transcription factor 1 (RUNX1)  
 Runx2, 608  
 Rural residence, 101  
 RXR. *See* Retinoid X receptor (RXR); Retinoid X receptor (RXR)  
 RYGB. *See* Roux-en-Y gastric bypass (RYGB)
- S**  
 S phase control, 757–758, 757f  
 S-adenosyl-methionine (SAM), 1152  
 S-adenosylhomocysteine (SAH), 1153–1154  
 S100 calcium-binding protein A11, 191  
 S100A11 pseudogene 3 (S100A11P3), 191  
 S100A11P3. *See* S100A11 pseudogene 3 (S100A11P3)  
 SACN. *See* Science Advisory Council for Nutrition (SACN); UK Scientific Advisory Committee on Nutrition (SACN)  
 Safety signals, 437  
 SAH. *See* S-adenosylhomocysteine (SAH)  
*Salmo salar*, 82, 84  
*Salmo salar*. *See* Atlantic salmon (*Salmo salar*)  
*Salmonella typhimurium*, 799–801, 1263  
 SALT. *See* Severity of Alopecia Tool (SALT)  
 SAM. *See* S-adenosyl-methionine (SAM)  
 Sanjad-Sakati syndrome, 309  
 SAPS II. *See* Simplified Acute Physiology Score II (SAPS II)  
 Sarcoidosis, 628, 631  
 Sarcopenia, 171  
 SARS-CoV-2, 1091  
   infection, 468–469, 1094  
   rate, 25  
   vitamin D and SARS-CoV-2 vaccination response, 1100  
 SARS-CoV2. *See* Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2)  
*Satb1* gene, 1141  
 Scalp psoriasis treatment, 1216  
 Scanning electron microscope (SEM), 229  
 SCCs. *See* Squamous cell carcinomas (SCCs)  
*Schistosoma mansoni*, 1060–1061  
 Science Advisory Council for Nutrition (SACN), 1104  
 SCL. *See* Stem-cell leukemia factor (SCL)  
 SCLC. *See* Small-cell lung cancer (SCLC)  
 Scleroderma, 1218  
 SCs. *See* Stem cells (SCs)  
 SCUP-h. *See* Skin Cancer Utrecht-Philadelphia-human (SCUP-h)  
*Scurfy* mutation, 1138  
 SD. *See* Standard deviation (SD)  
 SDDs. *See* Standard vitamin D doses (SDDs)  
 Seasonal studies, 515  
 Seasonal variation in vitamin D status, 414–415  
 Seasonality, 1045  
   and sun exposure, 1260–1261  
 Sebocytes, 1211  
 Sec23 homolog A, coat complex II component (SEC23A), 192–193, 213–215  
 SEC23A. *See* Sec23 homolog A, coat complex II component (SEC23A)  
 Seco-steroid hormone 1,25(OH) $_2$ D $_3$ , 679  
 Secondary bile acid LCA, 877  
 Secondary endpoints, 467  
 Secondary fracture prevention with vitamin D or calcium and vitamin D, 426  
 Secondary hyperparathyroidism (SHPT), 459–460, 462, 569, 587–588, 620  
   calcifediol treatment of secondary hyperparathyroidism associated with chronic kidney disease, 462–465  
   pathophysiology of, 462  
   role of 1,25(OH) $_2$ D deficiency in pathogenesis of secondary hyperparathyroidism in chronic kidney disease, 599f  
   vitamin D control, 599–602  
   skeletal health in CKD unrelated to attenuation of, 602–603  
 Secondary progressive phase multiple sclerosis (SPMS), 1167–1168  
 Secosteroids, 78  
 Secreted frizzled related protein-4 (Sfrp4), 293  
 Securin, 758  
 SELECT. *See* SELEnium and vitamin E Cancer prevention Trial (SELECT)  
 Selection bias, 1169  
 Selective estrogen receptor modulator (SERM), 66  
 SELEnium and vitamin E Cancer prevention Trial (SELECT), 918  
 SEM. *See* Scanning electron microscope (SEM)  
 Seocalcitol, 812, 909, 958  
 Separase, 758  
 Sepsis, 1265–1266  
 Sepsis-related Organ Failure Assessment score (SOFar), 1104  
 Sequential double-staining method, 1210–1211  
 Sequential Organ Failure Assessment (SOFA), 1261  
 Sequestration effect, 546  
 Ser180, 283–284  
 Serine-threonine kinases, 805–806  
 SERM. *See* Selective estrogen receptor modulator (SERM)  
 Serpin B1, 1214  
 SERPINB1 gene, 692

- Serum, 1241, 1260, 1266  
 Serum 1,25(OH)<sub>2</sub>D levels, 592  
 Serum 25-hydroxyvitamin D (25(OH)D), 47  
   and breast cancer aggressiveness/survival, 830  
   and breast cancer risk, 829–830  
   concentrations, 479, 670  
   effect of weight loss on, 493  
   heritability of serum 25(OH)D levels, 186  
   justification for serum 25(OH)D concentration >25 nmol/L, 1–2  
   UK scientific advisory committee on nutrition, 1–2  
   justification for serum 25(OH)D concentration >50 nmol/L, 3–5  
   European food safety agency and nordic guidelines, 5  
   US/Canada institute of medicine, 3–4  
   status of host, 466–467  
   United Kingdom with, 58f  
 Serum alkaline phosphatase activity, 249, 329  
 Serum calcium concentration, parathyroid hormone in acute defense of, 304  
 Serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in Paget's patients, 659  
 Serum CTx, 164  
 Serum levels of vitamin D, 949–950  
 Serum phosphate concentration, 284  
 Serum PTH, 253  
 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2), 1011  
 Severe hypercalcemia, 592  
 Severe hypomagnesemia, 310  
 Severity of Alopecia Tool (SALT), 1220–1221  
 Sex chromosomes, 1135  
 Sex hormone-binding globulin (SHBG), 172  
 Sex hormones, 1135–1136  
 Sex steroids, 172  
 Sexual dimorphisms, 1134–1135  
 Sexual dimorphisms implicate hormonally regulated, CD4<sup>+</sup>T cell intrinsic protective mechanism, 1137  
 Sfrp4. *See* Secreted frizzled related protein-4 (Sfrp4)  
 Shade, 102  
 Shadow rule of vitamin D levels, 102–103  
 Shark cartilage, 625–626  
 SHBG. *See* Sex hormone-binding globulin (SHBG)  
 Short-chain fatty acids, 1059  
 Short-term determinants of 25(OH)D (2), 38  
 SHPT. *See* Pathophysiology of secondary hyperparathyroidism (SHPT); Secondary hyperparathyroidism (SHPT)  
 Signal transducer and activator of transcription 3 (STAT3), 799, 805  
 Signaling and execution of monocytic differentiation induced by VDDs, 742–752  
   activation of VDR, 742–746  
   differentiation signaling pathways, 746  
 Signaling molecule, 11  
 Silibinin, 771–772  
 Simplified Acute Physiology Score II (SAPS II), 1261  
 Single cell analysis of VDR, CYP27B1, and CYP24A1 mRNA expression in human breast tissue, 833f  
 Single nucleotide polymorphisms (SNPs), 32–33, 185, 202, 353–354, 571–572, 863, 938, 1058, 1100, 1110, 1132, 1199  
 Single-anastomosis duodenoileal bypass, 162  
 Single-cell sequencing techniques, 680  
 Single-dose therapy, 253–254  
 Singlet oxygen, 80–81  
 Sizable brown adipose tissue, 497  
 Skeletal effects  
   of bariatric surgery, 163–166  
     bone mass, 164–165  
     bone microstructure, 165  
     bone turnover markers, 164  
     comparison with nonsurgical weight loss, 166  
     fracture risk, 166  
   prevention and treatment of, 175–177  
   management of osteoporosis after bariatric surgery, 177  
   skeletal health recommendations, 176t  
   universal recommendations, 175–177  
 Skeletal health, 175  
   in CKD unrelated to attenuation of secondary hyperparathyroidism, vitamin D control of, 602–603  
   metabolism and role of vitamin D on, 663–665  
 Skeletal muscle, 513–514  
   satellite cells, 741  
 Skin  
   aging, 1221  
   lesions treatment in children, 1216  
   pigmentation-related diseases, 189  
   tumors in mice after chronic UV exposure, 975f  
 Skin cancer, 719, 967–968, 972–975  
   mechanisms of protection from UV-induced skin tumors, 975–983  
   relationships, 973–975  
   similarity of action spectra for, 968  
   sun avoidance because of concern about, 103  
   synthesis and metabolism of, 972–973  
 Skin Cancer Utrecht-Philadelphia-human (SCUP-h), 968  
 Skin disease, 1223  
   vitamin D in prevention and therapy, 1218–1223  
     acne, 1222–1223  
     cutaneous wound healing and skin health, 1223  
     ichthyosis, 1218  
     scleroderma, 1218  
     vitamin D and autoimmune bullous diseases of skin, 1218–1220  
   “Skip” lesions, 1057–1058  
 sKlotho. *See* Soluble klotho (sKlotho)  
 Skull abnormalities, 246–247  
 Skull CT scan, 232f  
 SLC12A3 gene, 282, 312  
 SLC24A4 gene, 185–186  
 SLC34A1 gene, 282, 401  
 SLC34A2, 282  
 SLC34A3 mutations, 293  
 SLE. *See* Systemic lupus erythematosus (SLE)  
 Sleeve gastrectomy, 163–165, 169–170  
 Smad3, 696  
 SMAD3. *See* Small mothers against decapentaplegic 3 (SMAD3)  
 SMAD4, 951  
 Small cell carcinoma, 727  
 Small mothers against decapentaplegic 3 (SMAD3), 1018  
 Small nucleolar RNAs (sno RNAs), 691  
 Small-cell lung cancer (SCLC), 901–902  
 Smo signaling. *See* Smoothed signaling (Smo signaling)  
 Smoking, 201  
   tobacco, 1059  
 $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), 952  
 Smoothed signaling (Smo signaling), 973–974  
 SNAIL1, 866  
 SNAIL2, 866  
 sno RNAs. *See* Small nucleolar RNAs (sno RNAs)  
 SNPs. *See* Single nucleotide polymorphisms (SNPs)  
 SOCS. *See* Suppressor of cytokine signaling (SOCS)  
 Sodium excretion, 622  
 Sodium-dependent phosphate absorption, 281  
 Sodium-dependent phosphate cotransporter (NaPi-IIc), 252  
 SOFA. *See* Sequential Organ Failure Assessment (SOFA)  
 SOFAR. *See* Sepsis-related Organ Failure Assessment score (SOFAR)  
 Soft-tissue calcifications, 629  
 Soladek, 626, 639  
 Solar ultraviolet, 35, 99, 947, 967  
   nonvitamin D benefits from solar UV exposure, 107–108  
   solar UVB, 32, 97  
   exposure, 98  
   radiation, 939–947, 1076  
   vitamin D production from, 97–98  
 Solar zenith angle (SZA), 100  
 Soluble adenylate cyclase 10 (ADCY10), 621  
 Soluble klotho (sKlotho), 604–605  
 Soluble receptor, 1044  
 Solvents, 81  
 SORT1. *See* Sortilin 1 (SORT1)  
 Sortilin 1 (SORT1), 189

- Sotrovimab, 1095–1099
- South America, vitamin D status in, 49, 50t–54t
- SOX9 transcription factor, 806–807
- Spatiotemporal single-cell sequencing, 680
- Specificity protein 1 transcription factor, 754
- Speculation, 1242
- SPF. *See* Sun protection factor (SPF)
- Spike (S), 1091
- SPINK1* mutation, 938
- Spirocerca lupi*, 668–669
- Splice site mutation, 291
- Spline regression models, 544
- SPMS. *See* Secondary progressive phase multiple sclerosis (SPMS)
- Spontaneous healing, 385  
hypothesis regarding mechanism for, 385–387
- Sporadic CRC, 859
- Sprouty receptor tyrosine kinase signaling antagonist-2 (SPRY2), 868–869
- SPRY2. *See* Sprouty receptor tyrosine kinase signaling antagonist-2 (SPRY2)
- SQLE. *See* Squalene epoxidase (SQLE)
- Squalene epoxidase (SQLE), 701–702
- Squamous cell carcinoma of the lung (LSCC), 902
- Squamous cell carcinomas (SCCs), 759, 967, 1210–1211
- SR. *See* Systematic reviews (SR)
- SR/Mas. *See* Systematic reviews/metaanalyses (SR/Mas)
- SRC-1 and SRC-2. *See* Steroid receptor coactivators 1 and 2 (SRC-1 and SRC-2)
- SREBP-1c. *See* Sterol regulatory element-binding protein 1c (SREBP-1c)
- SRMs. *See* Standard reference materials (SRMs)
- Stability of vitamin D, 78–81  
antirachitic compounds vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in crystal form and dissolved in organic solvents, 78–79  
exposure to light, 79  
oxidants and singlet oxygen, 80–81  
solvents and vitamin D, 81  
thermal treatment, 79–80  
treatment at different pH, 80
- Stage I rickets, 249
- Standard deviation (SD), 213
- Standard Reference, 69
- Standard reference materials (SRMs), 666
- Standard vitamin D doses (SDDs), 98
- Staphylococcus aureus*, 799, 998, 1263
- Staphylococcus epidermidis*, 1263
- STAT3. *See* Signal transducer and activator of transcription 3 (STAT3)
- Statins, 127b
- Stem cells (SCs), 880
- Stem-cell leukemia factor (SCL), 752
- Stereoisomer, 664
- Steroid hormone, 560–561
- Steroid receptor coactivators 1 and 2 (SRC-1 and SRC-2), 978
- Steroids, 265
- Sterol regulatory element-binding protein 1c (SREBP-1c), 560
- Stimulation of proliferation, 701–702
- STK11* mutation, 938
- Stone disease, genetic contributions to, 619
- Stone formation, risk of, 619
- Streptococcus mutans*, 1002–1003
- Streptococcus pneumoniae*, 998, 1001–1002
- Streptozotocin (STZ), 1112
- Stress-activated protein kinases, 979
- Stroke, 570–571, 573–574, 577–578
- Stromal cell heterogeneity, 952
- Stromal cells, 657, 679
- Stromal remodeling, 679–680
- Study design, 721
- Study population 25(OH)D levels, 436–437
- “Stunned” para-thyroids, 310
- STURDY study, 436
- STZ. *See* Streptozotocin (STZ)
- Subarachnoid hemorrhage, 573–574
- Subcutaneous adipose tissues, 490
- Subperiosteal erosions, 252
- Subtilisin-like proprotein convertase, 283–284
- Sufficiency, 117
- Sulfate, 303
- Sulfotransferase family 2A member 1 (SULT2A1), 192
- SULT2A1. *See* Sulfotransferase family 2A member 1 (SULT2A1)
- Sun allergy, 982
- Sun avoidance, 105  
of concern about skin cancer, melanoma, 103
- Sun exposure, 117, 724–725  
endogenous characteristics, 101–102  
factors affecting 25-hydroxyvitamin D concentrations, 98–101  
lifestyle factors associated with reduced, 105  
measures of sun exposure as surrogate of vitamin D status, 34–35  
and MS risk, 1169  
nonvitamin D benefits from solar UV exposure, 107–108  
predicting vitamin D deficiency, 107  
vitamin D production from solar UVB exposure, 97–98
- Sun protection factor (SPF), 639–640
- Sunlight, 48, 967–968  
exposure, 69–70
- Sunscreens  
use of, 102  
and vitamin D status, 1221–1222
- Sunshine, 11
- SunSmart, 103
- Superanalogs, 1118–1119
- Superantigens, 1208
- Superoxide dismutase, 979
- Supplemental calcium and vitamin D on risk of fractures, RCTs of effects of, 451  
large randomized controlled trials of supplemental vitamin D and calcium on fractures, 452t
- Supplemental vitamin D and calcium on incident fractures, systematic reviews and metaanalyses of, 446
- Supplemental vitamin D on fractures, large randomized controlled trials of, 444t–445t
- Supplemental vitamin D on incident fractures, systematic reviews and metaanalyses of, 443–446
- Supplemental vitamin D *vs.* placebo on areal bone mineral density in high-risk populations, RCTs of, 448
- Supplemental vitamin D *vs.* placebo on areal bone mineral density in general population, RCTs of, 447–448
- Supplemental vitamin D *vs.* placebo on fractures in high-risk populations, RCTs of, 450–451
- Supplemental vitamin D *vs.* placebo on incident fractures in general population, RCTs of, 448–450  
incident fractures in vitamin D and placebo groups, 449f
- Supplemental vitamin D *vs.* placebo on volumetric bone mineral density, RCTs of, 448
- Supplementation, 494  
on obesity, 495
- Supplements, 116–117  
of Canadians, 121f  
country-level guidelines for supplements use, 118–120  
prevalence of inadequacy between supplement users and nonusers, 121t  
use improves vitamin D status, 120–123
- Suppressed immunestimulating cytokines, 972
- Suppression of parathyroid hormone secretion, 461–462
- Suppressor of cytokine signaling (SOCS), 1001–1002, 1018
- Surgeons, 162
- Surrogate of vitamin D status, sun exposure measures as, 34
- Survivin, 805
- Sustained virological response (SVR), 1012–1013
- SVR. *See* Sustained virological response (SVR)
- SW480-ADH colon cancer cells, 686
- Swedish study, 726–727
- Swordfish (*Xiphias gladius*), 88
- SXE motif, 291
- Symptomatic asthma, 1244
- Synaptophysin, 562–563
- Synovium, 1188
- Synthetic analogs, toxicity due to, 629–630

Synthetic vitamin D analogs, 774  
 alfacalcidol, 774  
 inecalcitol, 774  
 Systematic reviews (SR), 145–146  
 Systematic reviews/metaanalyses (SR/  
 Mas), 422–423  
 Systemic inflammation  
 conferring renal and cardiovascular  
 protection independently of renal  
 or systemic klotho levels, vitamin D  
 control of hypertension and,  
 606–609  
 vitamin D and, 514–515  
 Systemic klotho levels, vitamin D control  
 of hypertension and systemic  
 inflammation conferring renal and  
 cardiovascular protection  
 independently of, 606–609  
 Systemic lupus erythematosus (SLE), 2  
 SZA. *See* Solar zenith angle (SZA)

## T

T cell factor (TCF), 688–690, 861  
 T cell factor/lymphoid enhancer factor  
 (Tcf/Lef), 805–806  
 T cell receptor (TCR), 1021, 1036,  
 1040–1041, 1148  
 T cells, 1015–1016, 1130  
 effects of vitamin D upon  
 immunometabolism in, 1044–1045  
 immunoglobulin domain, 954  
 T cell-driven model, 1065  
 $\gamma\delta$ T cells, 798–799, 1040–1041  
 T follicular helper cells (TFH cells),  
 1019  
 T helper cells (Th cells), 798–799, 1038  
 T cell plasticity, 1040  
 Tfh cells, 1040  
 Th1 cells, 1038, 1092, 1130, 1146, 1208,  
 1210  
 Th2 cells, 1038–1039, 1077, 1092  
 Th9 cells, 1039  
 Th17 cells, 1039–1040, 1130, 1210  
 T lymphoblasts, 372–373  
 T lymphocytes, 767, 1035–1038, 1117–1118,  
 1130. *See also* B lymphocytes  
 T regulatory cell (Treg cell), 1130  
 T regulatory type-1 (Tr1), 1130  
 T-cell proliferation, 1243  
 T1D. *See* Type 1 diabetes (T1D)  
 T2D. *See* Type II diabetes (T2D)  
 TA cells. *See* Transit amplifying cells (TA  
 cells)  
 Tacalcitol, 1215  
 Tachysterol, 972–973  
 TACO. *See* Tryptophan aspartate-  
 containing coat protein (TACO)  
 Tacrolimus, 1119  
 TAF12 antisense construct on OCL  
 formation in marrow cultures from  
 PD patients expressing MVNP in  
 OCL precursors, effects of  
 transduction of, 655  
 TAF12 binds Cyp24A1 promoter in  
 osteoclast precursors, 655  
 TAF12 enhances vitamin D nuclear  
 receptor content and half-life in  
 cells, 656  
 stabilization of 1,25(OH)<sub>2</sub>D<sub>3</sub>, 657f  
 TAF12 expression by osteoclast  
 precursors, effects of, 654  
 TAF12 in increased 1,25(OH)<sub>2</sub>D<sub>3</sub>  
 responsivity of OCL precursors  
 from Paget's patients, mechanism of  
 action of, 655–656  
 TAF12 in mouse OCLs inhibits bone  
 destruction and bone-remodeling,  
 deleting, 657–658  
 expression levels of cathepsin K, LC3  
 and syntaxin 17 in osteoclasts  
 derived from Trap-Cre/Taf12<sup>fl</sup>/  
 mouse bone marrow cultures, 659f  
 femur sections from T-Cre(–)/Taf12<sup>fl</sup>/  
 and T-Cre(+)/Taf12<sup>fl</sup>-at 8-month-  
 old age stained with TRACP and  
 anti-STX17, 659f  
 osteoclast formation and bone  
 resorption by Trap-Cre/Taf12<sup>fl</sup>/  
 OCLs, 658f  
 Trap-Cre(+)/Taf12<sup>fl</sup>-mice exhibit  
 increased bone mass, and decreased  
 bone resorption, 658f  
 TAF12 in osteoclast precursors from  
 Paget's disease patients contribute  
 to hyperresponsivity to  
 1,25(OH)<sub>2</sub>D<sub>3</sub>, increased levels of,  
 652–655  
 TAF12 interacts with ATF7, 655–656  
 TAF12 sufficient to induce 1,25(OH)<sub>2</sub>D<sub>3</sub>  
 hyperresponsivity in human  
 osteoclast precursors,  
 overexpression of, 654–655  
 TAF12, measles virus infection of  
 osteoclast precursors induces, 653  
 TAF12, transduction of antisense measles  
 virus nucleocapsid protein  
 construct into osteoclast precursors  
 from patients with Paget's disease  
 reduced expression of, 654  
 TAF4b, 653  
 TAFs. *See* TBP-associated factors (TAFs);  
 Tumor-associated fibroblasts (TAFs)  
 TAG. *See* Triglyceride (TAG)  
 TAG formation. *See* Triacylglycerol  
 formation (TAG formation)  
 Tag SNPs, 354  
 TAL1. *See* Stem-cell leukemia factor (SCL)  
 TAMs. *See* Tumor-associated macrophages  
 (TAMs)  
*TaqI*, 1082–1083  
*TaqI* VDR polymorphism, 1084  
 TaqMan allelic-discrimination real-time  
 polymerase chain reaction, 1213  
 Tartrate-resistance acid phosphatase  
 (TRACP), 653  
 tartrate-resistance acid  
 phosphatase–driven measles virus  
 nucleocapsid gene expression in  
 OCLs express TAF12, transgenic  
 mice with, 653  
 Tartrate-resistant acid phosphatase-  
 Cremice (Trap-Cremice), 657–658  
 TATA-binding protein (TBP), 652  
 TB. *See* Tuberculosis (TB)  
 TBP. *See* TATA-binding protein (TBP)  
 TBP-associated factors (TAFs), 652  
 TCA. *See* Tricarboxylic acid cycle (TCA)  
 TCF. *See* T cell factor (TCF)  
 TCF-4, 696–697  
 Tcf/Lef. *See* T cell factor/lymphoid  
 enhancer factor (Tcf/Lef)  
 TCGA. *See* The Cancer Genome Atlas  
 (TCGA)  
*TCIRG1* gene, 235  
 TCR. *See* T cell receptor (TCR)  
 TEDDY. *See* The Environmental  
 Determinants of Diabetes in Young  
 (TEDDY)  
 TEL2. *See* ETV7 transcription factors  
 Telomerase activity, 691–692  
 Tender joint score (TJC), 1200  
 Tenofovir, 284–285  
 Teriparatide, 317–318  
 Termination codon (TAA), 374–375  
 Testosterone (TST), 171, 1135, 1139  
 Tetany, 237  
 Tfh cells. *See* Follicular helper cells (Tfh  
 cells)  
 TFH cells. *See* T follicular helper cells (TFH  
 cells)  
 TFIID complex. *See* Transcription  
 initiation factor IID complex (TFIID  
 complex)  
 TGF. *See* Transforming growth factor  
 (TGF)  
*Tgfb* gene, 1112, 1138  
 Th cells. *See* T helper cells (Th cells)  
 Th17. *See* Inflammatory T helper 17 (Th17)  
 THBD. *See* Thrombomodulin (THBD)  
 The Cancer Genome Atlas (TCGA), 837  
 The Environmental Determinants of  
 Diabetes in Young (TEDDY), 1110  
 The Obesity Society (TOS), 175–176  
 Therapeutic agents, 610  
 Thermal degradation, 79–80  
 Thiazide diuretics, 639–640  
 Thiazolidinedione Intervention with  
 vitamin D Evaluation (TIDE), 532  
 Thioredoxin (TRX), 691, 1264  
 Thioredoxin-interacting protein (TXNIP),  
 765, 1264  
 1000 genomes project, 349  
 Thr146Ile. *See* Threonine to isoleucine at  
 amino acid 146 (Thr146Ile)  
 Threonine to isoleucine at amino acid 146  
 (Thr146Ile), 380  
 Thresholds for 25(OH)D, 117–118  
 Thrombocytopenia, 669  
 Thrombomodulin (THBD), 746  
 Thymine, 101  
 Thymine–cytosine, 968–969  
 Thymus, 604  
 Thyroid cancer, 310  
 Thyroid hormone receptor–associated  
 polypeptide (TRAP), 753



- Thyroid receptor (TR), 374  
 Thyroparathyroidectomy, 637  
 Thyrotropin (TSH), 311  
 TIDE. *See* Thiazolidinedione Intervention with vitamin D Evaluation (TIDE)  
 Tight junctions (TJ), 1094, 1213  
 Tilapia (*Tilapia mossambica*), 84  
*Tilapia mossambica*. *See* Tilapia (*Tilapia mossambica*)  
 Time of flight, 1214  
 TIMP-1. *See* Tissue inhibitors of metalloproteinase-1 (TIMP-1)  
 TIO. *See* Induce osteomalacia (TIO); Tumor induced osteomalacia (TIO); Tumor-induced osteomalacia (TIO)  
 Tissue inhibitors of metalloproteinase-1 (TIMP-1), 687  
 Tissue-nonspecific alkaline phosphatase (TNSALP), 226, 603  
 Tissues, 7  
 TJ. *See* Tight junctions (TJ)  
 TJC. *See* Tender joint score (TJC)  
 TLRs. *See* Toll-like receptors (TLRs)  
 TME. *See* Tumor microenvironment (TME)  
 TMEM151A gene, 191  
 TmP. *See* Maximum tubular reabsorption rate for phosphate (TmP)  
 TMPRSS2 genes, 918  
 TNBC. *See* Triple-negative breast cancers (TNBC)  
 TNBS. *See* Trinitrobenzene sulfonic acid (TNBS)  
 TNF. *See* Tumor necrosis factor (TNF)  
*Tnfa*, 1112  
 TNF $\alpha$ . *See* Tumor necrosis factor  $\alpha$  (TNF $\alpha$ )  
 TNSALP. *See* Tissue-nonspecific alkaline phosphatase (TNSALP)  
 Tobacco smoke, 1235  
 Tocilizumab, 1095–1099  
 TolDCs. *See* Tolerogenic dendritic cells (TolDCs)  
 Tolerable upper intake level, 1271  
 Tolerogenic dendritic cells (TolDCs), 1044, 1186  
 Toll-like receptors (TLRs), 389, 867–868, 1016–1017, 1035, 1058, 1077, 1092, 1185–1186, 1262  
   TLR1, 995–996  
   TLR2, 995–996  
   TLR4, 995–996  
   TLR-mediated inflammation, 561–562  
 Tooth eruption, 247  
 Topical calcipotriol, 630  
 TOPORS antisense RNA 1 (TOPORS-AS1), 870  
 TOPORS-AS1. *See* TOPORS antisense RNA 1 (TOPORS-AS1)  
 TOS. *See* The Obesity Society (TOS)  
 Total parenteral nutrition (TPN), 263–264  
 Total thiol concentration (TTC), 878  
 Total vitamin D activity, 149–150  
   distribution of vitamin D  
   vitamins in food and calculation of, 89–91  
 Toxicity due to synthetic vitamin D analogs, 629–630  
 Toxicity of vitamin D II, 21  
 Toxicity threshold, 1271  
 TP53 gene, 873, 938, 951  
 TPA. *See* 12-O-tetradecanoylphorbol-13-acetate (TPA); Phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA)  
 TPA-responsive element (TRE), 746  
 TPN. *See* Total parenteral nutrition (TPN)  
 TR. *See* Thyroid receptor (TR); True retention (TR)  
 Tr1. *See* T regulatory type-1 (Tr1)  
 Trabecular bone, 229  
 Trabecular microstructural properties, 165  
 TRACP. *See* Tartrate-resistance acid phosphatase (TRACP)  
 TRACP–TAF12 transgenic mice hypersensitive to 1,25(OH) $_2$ D $_3$ , osteoclast precursors from, 655  
 Traditional food fortification with vitamin D, 143–144  
 Traditional risk markers, 574  
 TRAMP. *See* Transgenic adenocarcinoma of mouse prostate (TRAMP)  
 Transcellular calcium transport, 168  
 Transcription factor  
   p53, 755  
   in VDD-induced differentiation, 752–755  
   activator protein-1 transcription factor, 753–754  
   master transcription factors, 754  
   other transcription factors contribute to VDD-induced differentiation, 754–755  
   specificity protein 1 transcription factor, 754  
 Transcription initiation factor IIH complex (TFIIH complex), 971  
 Transcription-coupled repair, 971  
 Transcriptional start site (TSS), 1141  
 Transcriptomic approaches, 923  
 Transcriptomics of vitamin D, 1264–1265  
 Transcytosis, 605–606  
 $\beta$ -transducin repeat-containing protein ( $\beta$ -TrCP), 861  
 Transduction of antisense measles virus nucleocapsid protein construct into osteoclast precursors from patients with Paget's disease reduced expression of TAF12, 654  
 Transduction of TAF12 antisense construct on OCL formation in marrow cultures from PD patients expressing MVNP in OCL precursors, effects of, 655  
   osteoclast formation induced by 1,25(OH) $_2$ D $_3$  reduced in cultures of antisense TAF12, 656f  
 Transforming growth factor (TGF), 1021  
    $\beta$ 1, 1002  
   Smad signaling, 603  
   TGF  $\beta$ , 634, 695–696, 860, 1141  
 Transgenic adenocarcinoma of mouse prostate (TRAMP), 687  
 Transgenic mice with tartrate-resistance acid phosphatase-driven measles virus nucleocapsid gene expression in OCLs express TAF12, 653  
 Transgenic models of breast cancer, VDR agonists on, 840–841  
 Transient acantholytic dermatosis, 1223  
 Transient receptor potential vanilloid 5 (TRPV5), 304  
 Transit amplifying cells (TA cells), 863  
 TRAP. *See* Thyroid hormone receptor-associated polypeptide (TRAP)  
 Trap-Cremice. *See* Tartrate-resistant acid phosphatase-Cremice (Trap-Cremice)  
 TRE. *See* TPA-responsive element (TRE)  
 “Treat-to-target” concept, 543–545  
 Treg cell. *See* T regulatory cell (Treg cell)  
 Treg-specific demethylated region (TSDR), 1143  
 Tretinoin, 1222–1223  
 Triacylglycerol formation (TAG formation), 560  
 Trial to Reduce IDDM in Genetically at Risk (TRIGR), 1110  
 Trials, 1260  
 Tricarboxylic acid cycle (TCA), 1044  
*Trichinella spiralis*, 1060–1061  
 Triglyceride (TAG), 560  
 TRIGR. *See* Trial to Reduce IDDM in Genetically at Risk (TRIGR)  
 Trinitrobenzene sulfonic acid (TNBS), 1066  
 1,4,5-triphosphate, 1148  
 Triple-negative breast cancers (TNBC), 826  
 Trømso study, 511, 533–537, 541–543  
 Trousseau test, 305–306  
 Trout, 84  
 TRP. *See* Tubular reabsorption of phosphate (TRP)  
*Trp53*, 956  
*TRPM6* gene, 312  
 TRPV5. *See* Transient receptor potential vanilloid 5 (TRPV5)  
 True retention (TR), 87–88  
 TRX. *See* Thioredoxin (TRX)  
 Tryptophan aspartate-containing coat protein (TACO), 1076  
 TSDR. *See* Treg-specific demethylated region (TSDR)  
*TSFM* gene, 1131–1132  
 TSG. *See* Tumor suppressor genes (TSG)  
 TSH. *See* Thyrotropin (TSH)  
 TSKU. *See* Tsukushi, small leucine-rich proteoglycan (TSKU)  
 TSS. *See* Transcriptional start site (TSS)  
 TST. *See* Testosterone (TST)  
 Tsukushi, small leucine-rich proteoglycan (TSKU), 191  
*Tt* genotype, 1082–1083  
 TTC. *See* Total thiol concentration (TTC)  
 Tuberculosis (TB), 69, 632, 1018, 1048, 1075, 1231

- case series investigating effects of, 1080t  
 historical studies, 1079–1081  
 immune response in, 1076  
 influence of vitamin D on immune response to MTB, 1076–1079  
 studies of treatment outcome, 1083–1084  
 intervention studies, 1084  
 observational studies, 1083–1084  
 studies with active TB disease as end point, 1082–1083  
 intervention studies, 1083  
 observational studies, 1082–1083  
 studies with MTB infection as end point, 1081–1082  
 intervention studies, 1082  
 observational studies, 1081  
 vitamin D in prevention and treatment of tuberculosis, 1081f  
 Tuberculous pleuritis, 632  
 Tubular reabsorption of phosphate (TRP), 264  
 Tumor  
   stroma, 811  
   suppressor, 810–811  
 Tumor, 952  
 Tumor cells  
   apoptosis, autophagy, and energy metabolism, 693–695  
   cell cycle and DNA repair, 692–693  
   cell differentiation, 695  
   growth factors and growth factor receptors, 695–697  
   molecular action, 688–692  
   schematic representation  
     summarizing intracellular pathways and signaling pathways, 689f–690f  
   vitamin D effects on, 688–697  
   vitamin D resistance mechanisms in, 838–839  
 Tumor induced osteomalacia (TIO), 285t  
 Tumor initiating cells, 827  
 Tumor microenvironment (TME), 952  
   in pancreatic cancer, 952–954  
 Tumor necrosis factor (TNF), 1076, 1114  
 Tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), 171, 634, 799, 1058, 1099, 1190  
 Tumor suppressor, 692  
 Tumor suppressor genes (TSG), 688–692, 950–951  
 Tumor suppressor protein p53, role of, 980–981  
 Tumor-associated fibroblasts (TAFs), 806  
 Tumor-associated macrophages (TAMs), 927  
 Tumor-induced osteomalacia (TIO), 293  
 Tumor-related osteomalacia, 570  
 Tumoral calcinosis, 293–294  
 Twin-based studies, 186  
 TWIST1, 867  
 TXNIP. *See* Thioredoxin-interacting protein (TXNIP)  
 Type 1 diabetes (T1D), 2, 1109  
 immune effects of interventions with vitamin D in T1D, 1116–1118  
 interventions using vitamin D to prevent or arrest T1D, 1113–1116  
   animal studies, 1113–1115  
   human studies, 1115–1116  
 pathogenesis of, 1121f  
 relation between vitamin D–related genes and genetic predisposition to T1D, 1110–1111  
 vitamin D analogs and T1D, 1118–1119  
 vitamin D and beta cell, 1111–1113  
 vitamin D as immune modulator in, 1113  
 effect of vitamin D deficiency on T1Dpresentation, 1110  
 vitamin D in combination therapy to prevent or arrest T1D, 1119–1122  
 Type 2 diabetes, 135, 162, 175–176, 193, 213–214, 489, 511–512, 559, 797  
   biologic plausibility of association between vitamin D and, 512–515  
   insulin sensitivity, 513–514  
   mechanisms, 515  
   pancreatic beta-cell function, 512–513  
   systemic inflammation, 514–515  
   epidemiology and burden of, 511–512  
   importance of early-life vitamin D status for later obesity and subsequent risks of, 501–503  
   observational studies on vitamin D and, 515–526  
     case–control and cross-sectional studies, 515–516  
     limitations of observational studies and issue of confounding, 526  
     longitudinal cohort studies, 516–525  
     Mendelian randomization studies, 525–526  
     seasonal and geographic studies, 515  
   safety of vitamin D supplementation for prevention of, 546  
   vitamin D and, 537–538  
   vitamin D supplementation for  
     prevention people with prediabetes, 533–543  
     results from individual clinical trials, 533–539  
     results from individual participant data metaanalysis, 541–542  
     vitamin D and regression to normal glucose regulation, 543  
     vitamin D supplementation and regression to normal glucose regulation, 542–543  
 Type 2 diabetes mellitus (T2DM). *See* Type 2 diabetes  
 Type I glycogen storage disease, 284–285  
 Type I Na/Pi cotransporter, 282  
 Type II IGF receptor (IGF-R-II), 696, 873  
 Type II sodium phosphate cotransporter, 292  
 Type VI collagen receptor, 562–563  
 Tyr295stop mutation, 375–376  
 Tyrosine (TAC), 374–375  
 U  
 U.S. National Institutes of Health, 1233  
 UC. *See* Ulcerative colitis (UC)  
 UCP-1. *See* Uncoupling protein-1 (UCP-1)  
 UDP glucuronosyltransferase family 1 member A5 (UGT1A5), 190  
 UDP-N-acetyl-alpha-D-galactosamine, 294  
 UF. *See* Uncertainty factor (UF)  
 UGT1A5. *See* UDP glucuronosyltransferase family 1 member A5 (UGT1A5)  
 UK biobank data analysis, 503  
 UK National Diet and Nutrition Survey, 6, 148  
 UK Scientific Advisory Committee on Nutrition (SACN), 1–2, 117  
 UL. *See* Upper intake level (UL)  
 Ulcerative colitis (UC), 802–803, 859, 996, 1057–1058  
 Ultraviolet A (UV A), 1094, 1221–1222  
 Ultraviolet B (UVB), 32, 97, 115–116, 136–137, 185–186, 223, 413–414, 1045, 1094, 1208–1209  
   doses, 100  
   radiation, 34, 719, 1259  
   range, 589  
   UVB-exposure, 86  
   and vitamin D at skin, 1048–1049  
 Ultraviolet irradiation, 341  
 Ultraviolet light (UV light), 35, 48, 967  
   adaptation to, 968  
   irradiation, 153  
   mechanisms of protection from UV-induced skin tumors, 975–983  
     DNA repair proteins and processes, 981–982  
     potential contributors to reduced photocarcinogenesis, 983  
     reduced generation of reactive oxygen and nitrogen species, 979–980  
     reduction in DNA damage and enhancement of repair, 976  
     role of tumor suppressor protein p53, 980–981  
     role of VDR and receptors, 976–978  
     vitamin D compounds and UV-induced immune suppression, 982–983  
   messages to get UV exposure mornings and afternoons, 102–103  
   signature mutations, 969  
   skin tumors in mice after chronic UV exposure, 975f  
 UV-induced DNA damage, repair, and immune suppression, 968–972  
   direct DNA damage by absorption of UV, 968–969  
   DNA damage by UV exposure, 969f  
   DNA repair, 971–972  
   indirect DNA damage by UV, 969–970  
   UV-induced immune suppression, 972

- Ultraviolet light (UV light) (*Continued*)  
 UV-induced skin tumors, mechanisms of protection from, 975–983  
 Ultraviolet radiation (UVR), 5–6, 414, 828–829, 979  
 Uncertainty factor (UF), 128  
 Uncoupling protein-1 (UCP-1), 494  
 Unfractionated bone marrow cells, 657  
 United Kingdom  
   in National Dietary and Nutrition Survey, 55  
   with serum 25-hydroxyvitamin D, 58f  
 United States (US), 135–136, 443, 462, 724, 917  
 United States Centers for Disease Control, 511–512  
 3'-untranslated region (UTR), 867  
 UPP1. *See* Uridine phosphatase 1 (UPP1)  
 Upper intake level (UL), 3, 16, 21, 127, 138, 149–150, 1271  
 Upregulation  
   of CDKI levels, 760t  
   of DKK1, 869–870  
   of p21<sup>Cip1</sup> and p27<sup>Kip1</sup>, 759–762  
 Urban residence, 101  
 Uremia, analogs in animal models of, 569–570  
 Uridine phosphatase 1 (UPP1), 881  
 Urinary hydroxyproline excretion, 477  
 Urinary tract disease, 669–670  
 Urinary tract infections (UTI), 1003  
 Urine calcium, 249  
 US. *See* United States (US)  
 US Food and Drug Administration (FDA), 174, 318  
 US Institute of Medicine (IOM), 1, 3f  
 US National Academy of Medicine, 242–243  
 US National Health and Nutrition Examination Survey, 491  
 US National Institute for Standards and Technology, 1265–1266  
 US Preventive Services Task Force (2011) (USPSTF), 16, 443–446  
 US/Canada institute of medicine, 3–4  
 USPSTF. *See* US Preventive Services Task Force (2011) (USPSTF)  
 Utero “programming” of offspring, 502  
 UTI. *See* Urinary tract infections (UTI)  
 UTR. *See* 3'-untranslated region (UTR)  
 UV A. *See* Ultraviolet A (UV A)  
 UV index (UVI), 102–103  
 UV light. *See* Ultraviolet light (UV light)  
 UV resistance—associated gene (UVRAG), 982  
 UV-damaged DNA-binding protein (UV-DDB), 971  
 UV-DDB. *See* UV-damaged DNA-binding protein (UV-DDB)  
 UV-induced immune suppression, vitamin D compounds and, 968  
 UVB. *See* Ultraviolet B (UVB)  
 UVI. *See* UV index (UVI)  
 UVR. *See* Ultraviolet radiation (UVR)  
 UVRAG. *See* UV resistance—associated gene (UVRAG)  
 V  
 v-Raf murine sarcoma viral oncogene homolog B (BRAF), 902–903  
 Vaccine efficacy, 1045  
 Vaccine responses, translational aspects of vitamin D regulation of, 1045  
 Val346Met mutation, 379  
 Variant-to-function problem, 1133  
 Vascular calcification, 126, 291. *See also* Cardiovascular calcification  
 Vascular endothelial growth factor (VEGF), 224, 874  
 Vascular endothelium, 1076  
 Vascular function, 575–577  
 Vascular smooth muscle cells (VSMC), 569, 607–608  
 VDD. *See* Vitamin D dependency (VDD)  
 VDDR. *See* Vitamin D—dependent rickets (VDDR)  
 VDDR-1A. *See* Vitamin D—dependent rickets type 1A (VDDR-1A)  
 VDDR-2A, 360, 363, 670  
 VDDR-2A. *See* Vitamin D—dependent rickets type 2A (VDDR-2A)  
 VDDR-I. *See* Vitamin D—dependent rickets, type I (VDDR-I)  
 VDDR-II. *See* Vitamin D dependent rickets, type II (VDDR-II); Vitamin D-dependent rickets type II (VDDR-II)  
 VDDR1A. *See* Vitamin D hydroxylation—deficient rickets, type 1A (VDDR1A)  
 VDDR1B. *See* Vitamin D hydroxylation—deficient rickets, type 1B (VDDR1B); Vitamin D—dependent rickets, type 1B (VDDR1B)  
 VDDs. *See* Vitamin D derivatives (VDDs)  
 VDES. *See* Vitamin D endocrine system (VDES)  
 VDI. *See* Vitamin D insufficiency (VDI)  
 VDPP. *See* Vitamin D Pooling Project of Rarer Cancers (VDPP)  
 VDR, rationale to treat PCa by targeting, 920–923  
 VDR “knockout” mouse (VDRKO mouse), 496–497, 512, 742, 832  
 Vdr alleles, 562  
 VDR complex, 598, 602  
 VDR functions in prostate, high-dimensional data approaches to understanding, 923–925  
 VDR gene, 363, 1083, 1110, 1112, 1131, 1137  
   polymorphisms, 1241–1242  
   polymorphisms in VDR gene and cardiovascular outcomes, 574  
 VDR immunoreactivity score, 679–680  
 VDR induction of longevity gene, 604  
 VDR polymorphisms, 1238  
 VDR retinoic acid X-receptor (VDR RXR), 513–514  
 VDR RXR. *See* VDR retinoic acid X-receptor (VDR RXR)  
 VDR signalling and antiinflammatory responses in liver, 561–562  
   enhances methionine cycle, 1152–1153  
   enhances oligodendrocyte differentiation, 1150–1151  
   in liver fibrosis and NASH, 562–563  
 VDR SNPs, 574  
 VDR-knockout models, 412  
 VDR-mediated regulation of FGF23, 603  
 VDR-RXR heterodimerization  
   mutations affecting, 378–379  
   structural analysis, 378  
 VDREs. *See* Vitamin D response elements (VDREs)  
 VDRKO mouse. *See* VDR “knockout” mouse (VDRKO mouse)  
 VDRs. *See* Vitamin D receptors (VDRs)  
 VDSP. *See* Vitamin D Standardization Program (VDSP)  
 VDUP1. *See* Vitamin D—upregulated protein-1 (VDUP1)  
 VEGF. *See* Vascular endothelial growth factor (VEGF)  
 Velocardiofacial syndrome, 306–309  
 Venous thrombosis, 577–578  
 Vertical sleeve gastrectomy, 163f  
 Very-low-density lipoprotein (VLDL), 218  
 Very-low-density lipoprotein receptor (VLDLR), 560  
 Vessels, 567–568  
 Veterinary species, 663–664  
   measurement of vitamin D metabolites in, 665–666  
 VGF. *See* VGF nerve growth factor (VGF)  
 VGF nerve growth factor (VGF), 190  
 VICTORY. *See* Vitamin D and Cardiovascular Risk study (VICTORY)  
 ViDA trial. *See* Vitamin D assessment trial (ViDA trial)  
 ViDiKids, 1082  
 VIM. *See* Vimentin (VIM)  
 Vimentin (VIM), 907  
 Viosterol, 1207  
 Viral control, immunological mechanisms of, 1015  
 Viral diarrhea, 1014  
 Viral disease in epidemiological and interventional studies, association between vitamin D insufficiency and, 1012–1015  
 Viral hepatitis, 1012–1013  
 Viral infections, 1011, 1019–1020  
 Virtual bone biopsy, 165  
 Virus-induced cervical cancer, 1014  
 Viruses, 1,25(OH)<sub>2</sub>D and innate immune sensing of, 1016–1017  
 Visceral adipose tissues, 490  
 Visceral adiposity, 489, 493  
 VITAL. *See* Vitamin D and Omega 3 Trial (VITAL)  
 Vitamin A, 16, 1079–1081

- Vitamin C, 681
- Vitamin D, 2, 20, 77–78, 81, 97, 135, 150, 167–170, 185–186, 202, 242–243, 256–258, 288, 303, 341, 359, 382–383, 387, 412, 417, 425, 438–440, 443, 451, 496–501, 503, 511–514, 619, 625–626, 652, 663, 670, 687, 798, 959, 995, 1011, 1057, 1066–1067, 1091–1092, 1143, 1235–1248
- actions in cancer
- combination therapy, 697–699
  - resistance and vitamin D metabolism, 699–701
  - stimulation of proliferation, 701–702
  - vitamin D and cancer, 679–688
  - vitamin D effects on tumor cells, 688–697
- actions
- on B lymphocytes, 1043
  - on immune microenvironment and function of APCs, 1043–1044
  - on proinflammatory T lymphocyte responses, 1037
  - on regulatory T lymphocyte responses, 1041–1043
- adipose tissue as vitamin D reserve, 490–491
- alterations in renal 1,25(OH)<sub>2</sub>D synthesis and 25(OH)D and 1,25(OH)<sub>2</sub>D catabolism in
- alterations in vitamin D bioactivation to 25(OH)D in chronic kidney disease, 589–592
- defective renal 25(OH)D uptake limits renal calcitriol synthesis in CKD, 591f
  - systemic and parathyroid vitamin D metabolism and actions, 590f
- analytical methodology, 81–82
- principle for extraction, detection, and quantification of, 81–82
- antibacterial activity of, 997f
- antiinflammatory effects, 1185–1189
- association between vitamin D
- insufficiency and viral disease in epidemiological and interventional studies, 1012–1015
  - acquired immunodeficiency syndrome, 1012
  - COVID-19, 1014–1015
  - dengue fever, 1013–1014
  - herpes, 1013
  - human cytomegalovirus, 1013
  - mononucleosis, 1013
  - pulmonary diseases, 1014
  - viral hepatitis, 1012–1013
  - virus-induced cervical cancer, 1014
- and autoimmune bullous diseases of skin, 1218–1220
- behavioral determinants, 102–107
- and beta cell, 1111–1113
- biologic plausibility of association between type 2 diabetes and, 512–515
- mechanisms, 515
- vitamin D and insulin sensitivity, 513–514
- vitamin D and pancreatic beta-cell function, 512–513
- vitamin D and systemic inflammation, 514–515
- biology of vitamin D in pancreatic cancer, 954–957
- vitamin D and pancreatic tumor cells, 954–956
- vitamin D and pancreatic tumor microenvironment, 956–957
- bridging gap by, 143–148
- calculators, 130
- central role of renal calcitriol production in mineral and skeletal homeostasis, 588f
- chemical structure of vitamin D
- vitamers, 78
- and chemoprevention of NSCLC, 904–905
- in combination therapy to prevent or arrest T1D, 1119–1122
- combining vitamin D with immunotherapeutics, 908–909
- combining vitamin D with targeted therapeutics, 907–908
- community observational studies, 1095–1099
- selected studies investigating vitamin D deficiency and COVID-19, 1096t–1098t
- concentrations, 354
- condition leading to instability or stability of, 81t
- consideration of vitamin D–fortified foods, 148–150
- control of hypertension and systemic inflammation conferring renal and cardiovascular protection independently of renal or systemic klotho levels, 606–609
- antihypertensive properties of 1,25(OH)<sub>2</sub>D–VDR complex, 607f
- control of secondary hyperparathyroidism, 599–602
- pathogenesis of parathyroid hyperplasia and 1,25(OH)<sub>2</sub>D resistance in CKD, 600f
- role of 1,25(OH)<sub>2</sub>D deficiency in pathogenesis of secondary hyperparathyroidism in chronic kidney disease, 599f
- control of skeletal health in CKD unrelated to attenuation of secondary hyperparathyroidism, 602–603
- and COVID-19, 1094
- protective effect of 1,25(OH)<sub>2</sub>D against sars-cov-2 infection, 1094f
- and critical care patients, 1265–1272
- assays for vitamin D, 1265–1266
- current guidance of COVID-19 and vitamin D and future research, 1104
- current intakes of vitamin D in young and adult populations in North America and Europe and compare against dietary targets, 138–143, 139t–140t
- deficiency risk factors in critically ill, 1260–1261
- chronic disease, 1261
  - ethnicity, 1260
  - medication usage, 1261
  - seasonality and sun exposure, 1260–1261
  - severity of critical illness and organ failure, 1261
- deprivation, 305
- determinants of vitamin D levels from sun exposure
- determining optimal serum 25-hydroxyvitamin D concentrations for musculoskeletal health, 415–416
- dietary recommendations to meet status gap, 136
- dietary reference values/intakes for vitamin D, 137t
- dietary strategies for increasing vitamin D intake, 143–148, 151t–153t
- dietary supplements solution, 137–138
- disparity between association, translational, and clinical studies, 1045–1048
- downregulation of renin production, 606
- dyslipidemia and nonalcoholic fatty disease of liver, 501
- effects, 939f
- in experimental animals, 569–570
  - in inflammation-associated breast cancer, 810
  - in inflammation-associated colorectal cancer, 806–807
  - in inflammation-associated hepatocellular cancer, 813
  - in inflammation-associated pancreatic cancer, 811–812
  - in inflammation-associated prostate cancer, 808–809
  - modification of vitamin D supplementation by BMI, 545–546
  - on cardiovascular cells, 569
- effects of active vitamin D analogs on fractures, 427
- anabolic vitamin D analogs, 2MD, 427
  - vitamin D analogs, eldecalcitol, 427
- effects of polymorphisms in VDR and vitamin D–binding protein genes, 1100
- effects of supplementary vitamin D on RA, 1200–1201
- effects of vitamin D alone or calcium and vitamin D on bone mineral density and falls, 416–418
- effects of vitamin D on CD4D T cell subsets, 1038–1040
- effects of vitamin D on immunity and graft rejection, 475–476



Vitamin D (*Continued*)

- immune effects of vitamin D, 476
- role of vitamin D in allograft rejection, 475–476
- effects of vitamin D on skeleton, 412–413
- effects of vitamin D upon immunometabolism in APC and T cells, 1044–1045
- in end-stage organ failure, 578
- endogenous characteristics, 101–102
- epidemiologic associations between PCa and vitamin D metabolites, 918–920
- 25-Hydroxyvitamin D status and disparity of PCa in men of African genomic ancestry, 920
- epidemiological and evidence supporting role of vitamin D in immune disease, 1036
- epidemiological studies, 939–950, 940t–943t
- epidemiology of, 135
- epigenetic insights into MS pathogenesis, 1133–1134
- ethnicity, 948
- excess, 580
- extra-skeletal actions of vitamin D in HVDRR patients, 388–390
- factors affecting 25-hydroxyvitamin D concentrations, 98–101
- and falls, 436–438
- in food, 82–91
  - and calculation of total vitamin D activity, 89–91
  - farmed by man-fish, eggs, milk, meat mushrooms, and plants, 84–87
  - fortified food, 87
  - processing of food—effects on degradation of vitamin D, 87–89
  - from wild fish and animal, 82–84, 83f
- food-based strategies are required, 138
- and fracture risk, 438–440
  - BMD, 438
  - fractures, 438–440
- genetic studies on vitamin D and cardiovascular outcomes, 571–574
- genetics, 949
- and hair diseases, 1220–1221
- on health and disease
  - COVID-19, 217
  - direct acyclic graph showing illustrating, 202f
  - hypertension, 215
  - immune disorders, 216–217
  - list of Mendelian randomization studies for, 203t–212t
  - lung function and respiratory conditions, 216
  - mortality risk and aging, 217–218
  - musculoskeletal outcomes, 215–216
  - neuropsychiatric outcomes, 217
  - nonlinear MR studies, 218
  - other outcomes, 218
  - pregnancy-related outcomes, 217
- risk of cancers, 214
- risk of cardiovascular events, 214–215
- type 2 diabetes, 213–214
- health
  - advantages and disadvantages of parent vitamin D to restore, 460
  - comparative advantages of using calcifediol to restore, 460–461
  - randomized controlled trials
    - comparing effects of calcifediol vs. vitamin D<sub>3</sub> on heterogenous disease, 917–918
  - high-dimensional data approaches to understanding VDR functions in prostate, 923–925
  - homeostasis, 341
    - disorders, 670
  - hormone, 1130
  - hospital observational studies, 1099
  - hydroxylases, 864–868
  - 25-hydroxylase, 1059
    - activity, 342, 343t
    - CYP2R1 principal human, 342–345
  - and hypertension, 500–501
  - hypothesis, 1061
  - immune effects of interventions with vitamin D in T1D, 1116–1118
  - as immune modulator in T1D, 1113
  - and immunity and inflammation, 1091–1094
  - immunological mechanisms of viral control, 1015
- increased levels of TAF12 in osteoclast precursors from Paget's disease patients contribute to hyperresponsivity to 1,25(OH)<sub>2</sub>D<sub>3</sub>, 652–655
  - effects of IL-6 on TAF12 expression by osteoclast precursors, 654
  - measles virus infection of osteoclast precursors induces TAF12, 653
  - overexpression of TAF12 sufficient to induce 1,25(OH)<sub>2</sub>D<sub>3</sub> hyperresponsivity in transduction of antisense measles virus nucleocapsid protein construct into osteoclast precursors from patients with Paget's disease
    - reduced expression of TAF12, 654
  - transgenic mice with tartrate-resistance acid phosphatase-driven measles virus nucleocapsid gene expression in OCLs express TAF12, 653
- and inflammation, 799–802
- inflammation and hormonal adipokines, 498–499
- influence on immune response to MTB, 1076–1079, 1077f
- 1,25(OH)<sub>2</sub>D influences T helper cell profile, 1078f
- 1,25(OH)<sub>2</sub>D regulates innate inflammatory responses to mycobacterial infection, 1078f
- case series investigating effects of, 1080t
- vitamin D in prevention and treatment of tuberculosis, 1081f
- insulin resistance, insulin secretion, and glycemia, 499–500
- and insulin sensitivity, 513–514
- intake, 147, 254
  - studies, 728
- interplay between vitamin D and CD4<sup>+</sup> type 1 regulatory T cells in MS, 1145–1149
- intervention studies in patients with pancreatic cancer, 945t–946t
- interventional data, 1267–1270
- interventions using vitamin D to prevent or arrest T1D, 1113–1116
- intoxication, 570
- isomerization products formed by UVB-exposure, 80f
- lack of knowledge of benefits of, 104–105
- large randomized controlled trials of vitamin D supplementation, 427–428
- lessons from PCa clinical trials with vitamin D compounds, 925–927
- chemoprevention of PCa by vitamin D supplementation, 925–926
- chemotherapy of PCa with 1,25(OH)<sub>2</sub>D<sub>3</sub> and analogs and impact of genomic ancestry, 926–927
- in long-term maintenance of eucalcemia, 304–305
- main challenges in epidemiologic study of vitamin D and disease, 36–38
  - biases reduced or eliminated to extent possible, 37
  - confounding limited to extent possible, 37–38
  - studies ideally assess dose–response relationship, 37
  - time period for assessment of vitamin D status covered based on pathogenesis of disease end point of interest, 36–37
- maintenance of renal FGF23/klotho axis, 603–606
  - vitamin D maintenance of renal klotho contributes to renal and cardiovascular protection, 606f
- malabsorption, 313
- mechanism of action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on osteoclast formation, 652
- mechanisms in critical illness, 1262–1265
  - antimicrobial peptides, 1263–1264
  - metabolomics, transcriptomics, and epigenetics of vitamin D, 1264–1265
- mechanistic insight in breast cancer, 844–848, 849f
- mendelian randomization studies, 428
- metaanalyses, 423–425

- calcium plus vitamin D supplementation *vs.* placebo and total fracture, 424f
- metabolism, 78f, 403f, 620
  - cat, 670–671
  - dog, 666–670
  - measurement of vitamin D
    - metabolites in veterinary species, 665–666
  - metabolism and role of vitamin D on skeletal health, 663–665
  - mutated CYP24A1 in, 403–404
  - phosphate and, 284
  - and regulation with special reference to cardiovascular disease, 568–569
  - vitamin D metabolic pathways and activating and inhibiting factors associated with kidney stone formation, 621f
- metabolites, 664
- methods of assessing vitamin D status, 32–36
  - conceptual framework of assessing long term vitamin D status, 32–33
  - measure of genetic variation of circulating 25(OH)D, 36
  - measures of circulating 25(OH)D, 33–34
  - measures of predicted 25(OH)D levels, 35–36
  - measures of sun exposure as surrogate of vitamin D status, 34–35
  - measures of vitamin D intake, 34
- molecular pathways regulation by, 806
- and mouse models of RA, 1189–1190
- and MS, 1168–1169
  - genetics, 1131–1134
- nonvitamin D benefits from solar UV exposure, 107–108
- nuclear receptor, 651
- nutrition, 47
- observational and interventional studies, 827–832, 827t–828t
  - and breast cancer subtypes, 830
  - population studies, 827
  - supplementation trials in breast cancer prevention, 831–832
- observational data, 1266–1267
  - acute kidney injury, 1266–1267
  - acute lung injury, 1267
  - sepsis, 1266
  - vitamin D and critical illness outcomes, 1266
- observational studies on, 515–526
  - case–control and cross-sectional studies, 515–516
  - limitations of observational studies and issue of confounding, 526
  - longitudinal cohort studies, 516–525
  - Mendelian randomization studies, 525–526
  - seasonal and geographic studies, 515
- 1,25(OH)<sub>2</sub>D
  - and antimicrobial peptides in antiviral immunity, 1017–1018
  - and antiviral CD4 T cell immunity, 1019–1021
  - and antiviral CD8 T cell immunity, 1021
  - and antiviral cytokine and chemokine responses, 1018–1019
  - and autophagy, 998–999
  - and B cell responses to viruses, 1021–1022
  - and innate immune sensing of viruses, 1016–1017
  - and natural killer cell responses, 1019
  - and natural killer cells, 1002
  - and neutrophils, 1001–1002
  - and nonclassical immune cells, 1002
  - barrier integrity, and complement activation, 996–997
  - evolutionary aspects of 1,25(OH)<sub>2</sub>D induction of AMPs and PRRs, 1000–1001
  - induces antimicrobial peptides implicated in antibacterial immunity, 997–998
  - promotes expression of pattern recognition receptors and cytokines, 999–1000
  - regulates release of reactive oxygen species, 1000
  - in vivo* evidence for 1,25(OH)<sub>2</sub>D regulation of antibacterial innate immunity, 1002–1004
  - and 25(OH)D toxicity, 625–628
  - optimizing blood 25(OH)D levels and concept of “treat-to-target”, 543–545
  - oral intake of, 947–948
  - paracrine system, 1235–1236
  - potential physiologic basis for adverse effect of bolus doses and high daily doses of, 438
  - predicting vitamin D deficiency, 107
  - prevalence of low vitamin D status, 135–136
  - prevalence of vitamin D deficiency in critically ill patient, 1260
  - primary fracture prevention with vitamin D or calcium and vitamin D, 418–423
    - cumulative incident total, nonvertebral, and hip fractures, 421f
  - primary fracture prevention with calcium and vitamin D, 422–423
  - single high annual or monthly doses of vitamin, 420–422
    - effect of vitamin D alone on fractures, 418–420
  - protection from exacerbation of airway disease by, 1048
  - race, and MS risk, 1175
  - rationale to treat PCa by targeting VDR, 920–923
  - effects of vitamin D and analogs in preclinical models of prostate, 920–923
- RCTs of effects of supplemental calcium and vitamin D on risk of fractures, 451
- regimen, 1238–1239
- renal regulation of vitamin D endocrine system, 588–589
- repletion, 438
- resistance
  - mechanisms in tumor cells, 838–839
- and respiratory tract infections, 1094
- restriction, 639–640
- role of vitamin D genetic factors in osteoporosis and possible interactions with vitamin D therapy, 413–414
- role on non-skeletal health in cats, 670–671
- role on non-skeletal health in dogs, 667–670
  - cardiovascular diseases, 669
  - critical care, 670
  - dermatology, 670
  - endocrine diseases, 669
  - gastrointestinal diseases, 667–668
  - immune-mediated diseases, 669
  - infectious diseases, 668–669
  - inflammation, 670
  - oncology, 669
  - pancreatic and hepatic diseases, 668
  - urinary tract disease, 669–670
- safety, 425–427
  - calcium and vitamin D, 425–426
  - secondary fracture prevention with vitamin D or calcium and vitamin D, 426
  - vitamin D, 425
- safety considerations and prerequisite data required prior to initiating public health measures, 154–155
- safety of vitamin D supplementation for prevention of type 2 diabetes, 546
- seasonal variation in vitamin D status, 414–415
- serum/plasma levels of vitamin D, 949–950
  - circulating vitamin D–binding protein, 950
  - predicted levels of plasma 25(OH)D, 950
  - vitamin D insufficiency/deficiency in patients with PC, 950
  - vitamin D status, 950
- signalling, 998, 1000f
  - impact in animal models of breast cancer, 839–841
  - in liver fibrosis and NASH, 562–563
- similarity of action spectra for vitamin D synthesis, 968
- single high annual or monthly doses of, 420–422
- and skin aging, 1221
- solar ultraviolet-B radiation, 939–947

Vitamin D (*Continued*)

- sources of vitamin D and role of food in meeting nutritional requirements, 136–137
- stability of, 78–81
  - antirachitic compounds vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in crystal form and dissolved in organic solvents, 78–79
  - exposure to light, 79
  - oxidants and singlet oxygen, 80–81
  - solvents and vitamin D, 81
  - thermal treatment, 79–80
  - treatment at different pH, 80
- steroid hormone, 637–638
- structure of, 79t
- studies reporting on, 944t
- study designs of vitamin D, 38–43
  - case–control studies, 40–41
  - cohort studies, 39–40
  - ecologic studies, 38–39
  - interventional studies, 41–42
  - Mendelian randomization studies, 42–43
- sufficiency, 1036–1037, 1048, 1110
- system, 305, 972–975
  - genetic polymorphisms in, 682
  - mechanisms of protection from UV-induced skin tumors, 975–983
  - relationships, 973–975
  - supports oligodendrocyte myelin repair and replacement, 1149–1150
  - synthesis and metabolism of, 972–973
- system, 798
  - in normal and psoriatic skin, 1208–1211
- therapy, 327, 639
- toxicity, 625, 634–635
  - clinical manifestations of, 638–641
  - control of renal 1 $\alpha$ -OHase in, 636
  - diagnosis of, 639
  - mechanisms of, 634–638
  - role of vitamin D receptor in, 635–636
  - treatment of, 639–641
  - vitamin D–binding protein and level of free metabolite in, 637–638
- translational aspects of vitamin D
  - regulation of adaptive immunity–vitamin D and vaccine responses, 1045
- translational studies as window to study effects of vitamin D on adaptive immunology, 1036–1037
- treatment and supplementation of, 264t
- treatment of posttransplant bone loss
  - with vitamin D and analogs, 480–482
  - change in bone mineral density in renal transplant recipients treated with calcitriol or placebo, 480f
- as treatment option for inflammatory bowel disease, 1068
- type of, 125
- UVB and vitamin D at skin, 1048–1049
- vitamin D and hypertension, 500–501
- vitamin D and immunomodulation, 1015–1016
- vitamin D and prognosis in patients with PC, 957–958
  - CYP24A1 expression, 958
  - vitamin D levels, 957–958
  - vitamin D receptor, 958
  - vitamin D–binding protein levels, 958
- vitamin D and SARS-CoV-2 vaccination response, 1100
- vitamin D deficiency following organ transplant, 478–480
- vitamin D deficiency prior to organ transplant, 476–478
- vitamin D dose in critical illness, 1270–1271
- vitamin D intervention studies in patients with PC, 958–959
- vitamin D production from solar UVB exposure, 97–98
- vitamin D supplementation and COVID-19, 1100–1104, 1101t–1102t
- vitamin D supplementation for prevention of type 2 diabetes in people with prediabetes, 533–543
  - results from individual clinical trials, 533–539
  - results from individual participant data metaanalysis, 541–542
  - vitamin D and regression to normal glucose regulation, 543
  - vitamin D supplementation and regression to normal glucose regulation, 542–543
- vitamin D supplementation in asthma, 1046t–1047t
- vitamin D supplementation in people with normal glucose tolerance or type 2 diabetes, 526–533
- vitamin D supplementation safe, 1271–1272
- vitamin D-1 $\alpha$ -hydroxylase, 1059
- vitamin D/VDR signaling and antiinflammatory responses in liver, 561–562
- vitamin D–biofortified foods, 150–154
- and vitiligo, 1221
- Vitamin D analogs, 481–482, 625, 630, 639
  - and functional analysis, 768–769
  - alfacalcidol, 768
  - inecalcitol, 768–769
  - paricalcitol, 769
- future therapy using rationally designed, 385
- and inflammation, 799–802
  - effect on immune cells, 801–802
  - effect on inducers of inflammation, 799–801
  - effect on receptors and mediators of inflammation, 801
- modulation of cell cycle events by, 758–763
- and T1D, 1118–1119
- Vitamin D and CardiOvascular Risk study (VICTORY), 447
- Vitamin D and Longevity trial, 729
- Vitamin D and Omega 3 Trial (VITAL), 41, 117, 447–451, 539, 577–578, 723, 1200–1201
- Vitamin D assessment trial (ViDA trial), 416, 436, 447, 499, 577
- Vitamin D compounds, 683, 980
  - in combination with agents, 770–772
  - antiinflammatory agents, 771
  - cytotoxic and cytostatic drugs, 770
  - kinase inhibitors, 771
  - phytochemicals, 771–772
  - protein kinase C activators, 771
  - retinoids, 770–771
  - and UV-induced immune suppression, 982–983
- Vitamin D deficiency, 1, 47–48, 58, 66, 107, 115–116, 135, 167, 193, 223, 228–231, 242–243, 245–246, 254, 260–262, 341, 412, 414, 443, 476–477, 569, 587–588, 606, 627–628, 802, 1002–1003, 1057, 1061, 1079, 1081–1082, 1259
  - effect on T1D presentation, 1110
  - epidemiology of, 242–246
  - and nutritional rickets in infants and children
    - biochemical abnormalities, 248–249
    - biomarkers of active rickets, 249–250
    - clinical presentation, 246–248
    - dietary calcium deficiency, 258–263
    - epidemiology of vitamin D deficiency and nutritional rickets, 242–246
    - graphic representation of synergistic effects of low dietary calcium and vitamin D status, 245f
    - growth plate in rickets, 252
    - historical perspective, 242
    - pathogenetic spectrum of nutritional rickets, 265–268
    - prevention, 254–258
    - radiologic changes, 251–252
    - recommended vitamin D<sub>3</sub> doses, 253t
    - rickets of prematurity or metabolic bone disease of prematurity, 263–265
    - serum alkaline phosphatase in diagnosing active rickets in children, 257f
    - total parenteral nutrition, 263–264
    - treatment, 252–254
    - treatment and supplementation of calcium, phosphate, and vitamin D, 264t
  - obesity and, 491–492
  - organ transplant, 478–480
    - vitamin D deficiency at time of organ transplantation, 478
    - vitamin D deficiency in long-term transplant recipients, 478–480
  - predicting vitamin D deficiency, 107
  - and prevalence of vitamin D deficiency in Australia and New Zealand, 67t–68t

- and RA disease risk, severity, and progression, 1190–1199, 1191t–1195t
- rickets  
 infant with, 247f  
 radiographic features of, 251f  
 prior to organ transplant, 476–478  
 risk factors for vitamin D deficiency in organ transplant patients, 477t  
 vitamin D deficiency in patients with chronic kidney disease, 478  
 vitamin D deficiency in patients with congestive heart failure, 477  
 vitamin D deficiency in patients with end-stage pulmonary disease, 476–477  
 vitamin D deficiency in patients with liver failure, 477
- vitamin D status  
 in Africa, 58  
 in Asia, 55  
 assessment of, 48–49  
 in Europe, 55  
 in Middle East, 55  
 in North America, 49  
 in Oceania, 66  
 in South America, 49, 50t–54t  
 study selection, 49–66
- Vitamin D dependency (VDD), 327
- Vitamin D dependent rickets, type II (VDDR-II), 361
- Vitamin D derivatives (VDDs), 741  
 cellular models of differentiation induced by 1,25(OH)<sub>2</sub>D<sub>3</sub> and, 743t–745t  
 clinical applications against hematopoietic malignancies, 768–774  
 effects on cell cycle and proliferation of human leukemia cells, 755–764  
 features of cell cycle, 755–756, 756f  
 regulation of cell cycle progression, 756–758  
 effects on cell survival and cell death, 764–765  
 effects on lymphoid lineage cells, 765–768  
 modulation of cell cycle events by vitamin D and analogs, 758–763  
 molecular targets of VDDs in leukemic cells, 748t–749t  
 signaling and execution of monocytic differentiation induced by, 742–752  
 significance of basic and clinical studies of VDDs in hematopoietic malignancies, 774–775  
 transcription factors in VDD-induced differentiation, 752–755  
 VDDs–induced progression of monocytic to macrophage differentiation, 751
- Vitamin D endocrine system (VDES), 587, 1211, 1222  
 renal regulation of, 588–589
- Vitamin D External Quality Assessment Scheme (DEQAS), 47, 666
- Vitamin D hydroxylation–deficient rickets  
 affected genes and clinical manifestations in VDDR 1B, 1A, and 2A, 328t  
 biochemical findings, 329  
 clinical manifestations, 328–329  
 evolution of VDDR1A under treatment from childhood to adulthood, 332–335  
 adult patients with VDDR1A, 333  
 from childhood to adulthood, 332–333  
 pregnancies in women with VDDR1A, 333–335  
 short-term effects of treatment with calcitriol, 332  
 genetic and molecular studies, 329–330  
 treatment of VDDR1A, 330–332
- Vitamin D hydroxylation–deficient rickets, type 1A (VDDR1A), 327, 329, 1132  
 adult patients with VDDR1A, 333  
 auxological, biochemical, and radiological data of adult patients with, 335t  
 evolution of VDDR1A under treatment from childhood to adulthood, 332–335  
 height according to age of onset of calcitriol treatment in adult patients with, 335f  
 mutations in *CYP27B1*, 331t–332t  
 permanent incisors, 334f  
 pregnancies in women with VDDR1A, 333–335  
 radiographs, 334f  
 treatment of VDDR1A, 330–332
- Vitamin D hydroxylation–deficient rickets, type 1B (VDDR1B), 327–328
- Vitamin D I  
 issues with reaching 25(OH)D thresholds for specific groups, 5–7  
 justification for serum 25(OH)D concentration >25 nmol/L, 1–2  
 justification for serum 25(OH)D concentration >50 nmol/L, 3–5  
 other issues surrounding definition of thresholds for vitamin D, 7
- Vitamin D Individual Patient Analysis of Randomized Trials (DIPART), 443–446
- Vitamin D insufficiency (VDI), 48, 69, 105, 457, 462, 477, 1013, 1130  
 with calcifediol treatment of, 460–462  
 advantages and disadvantages of parent vitamin D to restore vitamin D health, 460  
 comparative advantages of using calcifediol to restore vitamin D health, 460–461  
 pathophysiology of endocrine vitamin D insufficiency, 460  
 suppression of parathyroid hormone secretion, 461–462
- early genetic support for vitamin D insufficiency in MS pathogenesis, 1131–1132
- Mendelian randomization studies  
 establish vitamin D insufficiency as causal for MS, 1132–1133  
 treatment of vitamin D insufficiency in chronic kidney disease, 462–465  
 effects of bolus IV immediate-release calcifediol, 464f
- Vitamin D metabolite ratio (VMR), 665
- Vitamin D nanomulsion (NVD), 872
- Vitamin D pathway, 193  
 genetic variation and gene expression in, 730–732  
 normal and neoplastic breast cells/tissues deregulation of vitamin D pathway in breast cancer, in, 832–839  
 deregulation of vitamin D pathway in breast cancer, 837–838  
 local metabolism of vitamin D in mammary gland, 836–837  
 mechanisms of vitamin D resistance in tumor cells, 838–839  
 VDR expression and function in normal mammary gland, 832–836  
 in tumor-infiltrating cells and extracellular matrix, 848  
 vitamin D pathway-related polymorphisms, 1238
- Vitamin D Pooling Project of Rarer Cancers (VDPP), 722–723
- Vitamin D receptor interacting protein (DRIP), 753
- Vitamin D receptors (VDRs), 225, 243–244, 288, 304–305, 327–328, 342–344, 359, 364, 411–412, 457, 490, 512–514, 569, 587, 620–621, 634–635, 664, 679–682, 731, 798, 827, 903–904, 968, 995–996, 1012, 1015–1016, 1036, 1076, 1091–1092, 1109, 1130, 1174, 1185–1186, 1209–1210, 1231, 1259
- activation  
 as initial VDD-induced signal for differentiation, 742–746  
 arrangement of chromosomal gene and domains of, 365f  
 colorectal cancer, 864–868  
 compound heterozygous mutations in VDR, 380  
 effects of polymorphisms in VDR and vitamin D–binding protein genes, 1100  
 epidemiology, 680–682  
 expression  
 and function in normal mammary gland, 832–836  
 genetic polymorphisms in vitamin D system, 682  
 HVDRR without mutations in VDR, 381–382



- Vitamin D receptors (VDRs) (*Continued*)
- mutations causing premature termination of VDR, 374–377
  - deletions, 376–377
  - mutations that disrupt mRNA splicing, 376
  - nonsense mutations, 374–376
  - mutations in VDR, 381
  - mutations in VDR DNA binding domain, 374
  - mutations in VDR ligand-binding domain, 377–380
  - mutations affect 1,25(OH)<sub>2</sub>D<sub>3</sub>-binding, 377–378
  - mutations affect coactivator-binding, 379–380
  - mutations affect VDR-RXR heterodimerization, 378–379
  - role in vitamin D toxicity, 635–636
  - role of VDR and receptors, 976–978
  - serial link among, 465–466
  - splice site, duplication, and deletion mutations in, 375f
  - Vdr-deficient mice, 885
  - VDR-β-catenin protein–protein interaction enhancement, 868
- Vitamin D response elements (VDREs), 364–365, 466, 596–597, 652, 687–688, 742, 997, 1015–1016, 1059, 1130, 1141–1143, 1209–1210, 1263
- Vitamin D Standardization Program (VDSP), 47, 666
- standardization according to, 48f
- Vitamin D status, 47–49, 115–116, 137–138, 185, 516, 526, 590–591, 668–669, 1061, 1134–1135
- assessment of, 48–49
    - in Africa, 58
    - in Asia, 55
    - in Europe, 55
    - in Middle East, 55
    - in North America, 49
    - in Oceania, 66
    - in South America, 49, 50t–54t
  - in autoimmune bullous diseases, 1219
  - and cardiovascular outcomes in observational studies, 570–571
  - graphic representation of synergistic effects of, 245f
  - and prevalence of vitamin D deficiency in Australia and New Zealand, 67t–68t
  - sunscreens and, 1221–1222
  - and synovial immune cell function, 1187f
  - trends in, 69
- Vitamin D supplementation, 6, 137–138, 213, 255–256, 411–412, 511, 533, 576–577, 621, 1078–1079, 1240
- and kidney stone risk, 622
  - in people with normal glucose tolerance or type 2 diabetes, 526–533
  - prevent, or reduce, obesity, 493–495
  - RCTs of effects of
    - vitamin D supplementation on bone density, 447–448
    - vitamin D supplementation on fracture risk, 448–451
  - strategies, 610
  - studies, 574–577
  - trials, 580
  - trials in rheumatoid arthritis, 1197t–1198t
  - vitamin D effects on biochemical risk markers, 574–575
  - inflammation parameters, 575
  - lipid parameters, 574–575
  - PTH and FGF23, 575
  - renin–angiotensin–aldosterone system, 575
  - vitamin D effects on parameters of cardiovascular function, 575–577
  - vascular and cardiac function, 575–577
- Vitamin D supplements, 106, 241–242, 438–439, 724
- categories of, 116t
  - characteristics of vitamin D supplement users, 123–124
  - considerations choosing to take, 124–127
    - in Canada, 127t
    - coingestion with another micronutrient, 126
    - coingestion with lipid source, 125–126
    - product quality, 126–127
    - sources of vitamin D<sub>3</sub>, 125
    - taking vitamin D supplements daily *vs.* bolus dosing, 126
    - type of vitamin D, 125
  - country-level guidelines for supplements use, 118–120
    - examples of advice and/or recommendations, 119t
  - guidance needed for use of, 129–131
  - intake of vitamin D, 123t
  - safety of, 127–129, 128t
  - supplement use improves vitamin D status, 120–123
  - thresholds for 25(OH)D, 117–118
  - use of, 129t
- Vitamin D-dependent rickets type II (VDDR-II), 359–360
- Vitamin D-fortified foods, 106
- consideration of, 148–150
  - 25-hydroxyvitamin D metabolites, 149–150
  - in relation to forms of vitamin D and addition amounts for traditional fortification, 149
  - vitamin D2 *vs.* vitamin D3, 149
- Vitamin D<sub>2</sub>, 78–79, 125, 149, 627
- cancer, 17–19
  - cardiovascular, 20–21
  - COVID-19, 22–26, 25f
  - human biology, 14–15
  - mortality, 19–20
  - multiple sclerosis, 21–22
  - boxplots showing serum 25(OH)D levels, 22f
  - power calculation arising from MSociety workshop, 23f
  - nutrition clinical trials cannot match quality of evidence achieved with drugs, 15–17
  - baseline and follow-up 25(OH)D concentrations and vitamin D dosing regimens, 18t
  - random effects metaanalysis of clinical trials of, 24f
  - terminology of guidance values as apply to, 12f
  - toxicity, 21
- Vitamin D<sub>3</sub>, 78–79, 149, 589–590, 627, 798, 958, 1221, 1259
- sources of, 125
  - in soybean oil, 88
  - supplementation trials in MS, reflections on, 1155–1156
- Vitamin D-binding protein (DBP), 341, 413, 477, 492–493, 571–572, 591, 637, 664, 686, 798, 837, 918, 972–973, 996–997, 1012–1013, 1076, 1099, 1110, 1174, 1196, 1208–1209, 1238
- circulating, 950
  - effects of polymorphisms in VDR and vitamin D-binding protein genes, 1100
  - and level of free metabolite in vitamin D toxicity, 637–638
- Vitamin D-biofortified foods, 150–154
- Vitamin D-deficient rickets, 252–254
- Vitamin D-dependent rickets (VDDR), 232–233, 345–352
- genetic forms of, 313–314, 319
  - deficiency of 1α-OHase, 313–314
  - genetic causes of increased vitamin D metabolism, 314
  - hereditary resistance to 1,25(OH)<sub>2</sub>D, 314
- Vitamin D-dependent rickets, type 1B (VDDR1B), 244
- Vitamin D-dependent rickets, type I (VDDR-I), 327, 360–361
- Vitamin D-dependent rickets type 1A (VDDR-1A), 666
- Vitamin D-dependent rickets type 2A (VDDR-2A), 327–328, 359–360, 666
- Vitamin D-mediated suppression of hypertension, 609
- Vitamin D-regulated proteins, 919
- Vitamin D-related genes and genetic predisposition to T1D, relation between, 1110–1111
- Vitamin D-upregulated protein-1 (VDUP1), 691
- Vitamin E, 681
- Vitamin K, 667–668
- Vitamin K1, 959
- Vitamin K2, 959
- Vitamin supplement, 123–124
- Vitiligo, 1221

VLDL. *See* Very-low-density lipoprotein (VLDL)  
 VLDLR. *See* Very-low-density lipoprotein receptor (VLDLR)  
 VMR. *See* Vitamin D metabolite ratio (VMR)  
 Volume overexpansion, 303–304  
 Volumetric bone mineral density, RCTs of supplemental vitamin D *vs.* placebo on, 448  
 “Volumetric dilutional” effect, 515, 546  
 Von Jaksch–Luzet syndrome, 248  
 VSMC. *See* Vascular smooth muscle cells (VSMC)

## W

Walker carcinoma, 688  
 Warburg effect, 1044  
 Warburg effect/aerobic glycolysis, 694  
 WAT. *See* White adipose tissue (WAT)  
 WBS. *See* Williams–Beuren syndrome (WBS)  
 WD. *See* Western-style diet (WD)  
 Wee1 expression, 758  
 Weight loss  
   bariatric surgery, 161  
   effect of weight loss on serum 25(OH)D concentrations, 493  
 Western blot analysis, 1214  
 Western-style diet (WD), 881–882  
 Wet nursing, 242  
 Wheezing, 1014  
 WHI. *See* Women’s Health Initiative (WHI)  
 White adipose tissue (WAT), 560  
 White blood cells, 745  
 White button mushroom (*Agaricus bisporus*), 84, 86  
 WHO. *See* World Health Organization (WHO)  
 Whole exome sequencing, 363  
 Whole-genome sequencing, 691  
 Wild fish, vitamin D in food from, 82–84  
 Wild-type (WT), 654  
   littermates, 654  
   VDR, 363  
 Williams–Beuren syndrome (WBS), 402, 630  
 Wilson’s disease, 284–285  
 Winter hypothesis, 502  
 Winter-related AD, 1248  
 Wnt inhibitors, 602  
 Wnt signaling, 605  
 Wnt/ $\beta$ -catenin pathway, 805–806  
 Wnt/ $\beta$ -catenin signaling pathway, 751  
   antagonism by 1,25(OH) $_2$ D $_3$  in colon cancer, 868–871

  in animal models and clinical trials, 870–871  
   enhancement of VDR- $\beta$ -catenin protein–protein interaction, 868  
   induction of E-cadherin, 868–869  
   regulation of DKK1 and DKK4, 869–870  
 Women with VDDR1A, pregnancies in, 333–335  
 Women’s Health Initiative (WHI), 439, 494, 516–524, 720, 831  
   study, 728  
   trial, 446  
 Women’s Health Study, 516  
 World Health Organization (WHO), 143, 542, 1011, 1075  
 Worldwide vitamin D status  
   assessment of vitamin D status, 48–49  
   in Africa, 58  
   in Asia, 55  
   in Europe, 55  
   in Middle East, 55  
   in North America, 49  
   in Oceania, 66  
   in South America, 49, 50t–54t  
   ethnicity/migration, 66  
   implications, 69–70  
   multicenter and global studies using central laboratory facility, 66  
   nutrition, 66  
   risk groups, 66  
   standardization according to VDSP, 48f  
   study selection, 49–66  
   trends in vitamin D status, 69  
 Wound healing, 1223  
 WT. *See* Wild-type (WT)

## X

X-linked hypophosphatemia (XLH), 229, 233, 284–290, 285t, 329, 360–361  
   pathophysiology of, 286–288  
   rickets, 252  
   treatment of, 288–290  
 X-linked recessive hypoparathyroidism, 309  
 X-rays, 236–237  
   studies, 361  
 Xenografts, 883  
   prostate cancer PC-3, 688  
   xenografted mice, 883–884  
*Xenopus laevis*, 292  
 Xeroderma pigmentosum (XP), 971  
 Xeroderma pigmentosum  
   complementation group A protein (XPA), 971

Xeroderma pigmentosum  
   complementation group C protein (XPC), 971  
 Xeroderma pigmentosum  
   complementation group D protein (XPD), 971  
 Xeroderma pigmentosum  
   complementation group G protein (XPG), 971  
*Xiphias gladius*. *See* Swordfish (*Xiphias gladius*)  
 XLH. *See* X-linked hypophosphatemia (XLH)  
 XP. *See* Xeroderma pigmentosum (XP)  
 XPA. *See* Xeroderma pigmentosum complementation group A protein (XPA)  
 XPC. *See* Xeroderma pigmentosum complementation group C protein (XPC)  
 XPD. *See* Xeroderma pigmentosum complementation group D protein (XPD)  
 XPG. *See* Xeroderma pigmentosum complementation group G protein (XPG)

## Y

Young adult female, 242  
 Young children with rickets, 248  
 Young populations in North America and Europe and compare against dietary targets, current intakes of vitamin D in, 138–143

## Z

ZEB1, 867  
 ZEB2, 867  
 Zellweger syndrome, 189  
 ZFP36L2, 753  
 Zinc and ring finger 3 (ZNRF3), 861  
 Zinc finger protein 36 (ZPF36), 867  
 Zinc metalloproteinases, 286–287  
 Zincamino chelate, 959  
 ZNRF3. *See* Zinc and ring finger 3 (ZNRF3)  
 ZO-1. *See* Zonula occludens-1 (ZO-1)  
 ZO-2. *See* Zonula occludens-2 (ZO-2)  
 Zoledronic acid, 411–412  
 Zonula occludens-1 (ZO-1), 874, 1061  
 Zonula occludens-2 (ZO-2), 874  
 Zooplankton, 84  
 ZPF36. *See* Zinc finger protein 36 (ZPF36)



# Vitamin D

## Volume Two: Disease and Therapeutics

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